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The Ohio State University

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DISSERTATION

Presented in Partial Fulfillment of the Requirements for The Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By


* * * * *

The Ohio State University

1986

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Approved by:

Adviser
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DEDICATION

Dedicated to the quintessence of my existence .... my families.
ACKNOWLEDGEMENTS

As with most things in life, the longer you live the more experiences and attributes you accumulate and if you are honest with yourself, you realize that the source of these is usually from people, who care, and love to learn. It is to these people that I owe my personal and professional development to, for in many respects you are a sum of your previous experiences, which in my case has been due to the sincere efforts associated with the following people.

I express sincere appreciation to my adviser, Dr. Richard G. Olsen, who has been the cornerstone in my professional development. His intellectual impact on me, which started in the classroom and at the laboratory bench, will continue throughout my comparative medical career. It was with his innovative and educationally flexible attitudes that have allowed me to sample a tremendous cross-section of technical experiences throughout this program. This sincere appreciation is extended to the rest of my committee; Professor Dr. Capen, our Departmental Chairman; Professor Dr. Steven Krakowka; Assistant Professor Dr. Lawrence Mathes and members of the Graduate Committee, Professor Dr. Steven Weisbrode, and Professor Dr. Gary Kociba, all of whom have taught me comparative and veterinary medicine and exhibited such sound support and faith in me during the development and implementation of this unusual research program. I
hope I have met your expectations and have allowed this unique and rewarding research opportunity to remain a viable avenue for other future comparative medical students.

This unique research opportunity would not have been possible without the interactions of so many quality people. I recognize Dr. Peter J. Fischinger, Deputy Director of the National Cancer Institute, for allowing me an opportunity to study with him in his laboratory the past two years. The tremendous demand on his time by his pivotal position in the world's greatest cancer research facility did not detract from his willingness to challenge, teach, and provide me with unique insights into cancer research at a national and international level. It is for this comprehensive aspect of my training and its commitments, that I extend my deepest personal gratitude. My program would have failed miserably without the daily interactions of the Section Chief, Dr. William G. Roby, who provided an inexhaustive source of experienced retroviral biochemistry, aspects of experimental approaches, reviewership, and the warm and caring atmosphere provided to me and my family at various times by him and his wonderful wife Cynthia and son Tom. The laboratory technical help provided to me was more than just an outstanding pair of hands and laboratory experience, it involved the development of friendships, which always makes new experiences and learning more enjoyable. To Tim, Audry, and soon to be married son Randy, Demarais, Mike, Barbara, and son John Poore, and Miss Nancy Dunlop, I extend my sincerest thanks and hope for only the best in your future
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I simply recognize those to whom this dissertation is dedicated by stating that without the guidance, love and discipline given to me by my mother and father, I would not be a participant in the most beautiful aspect of life .... husband to the most unselfish, warm-hearted, caring, maternal woman I will ever know on this earth, and father of two of the most delightful girls I could ever hope to share my life with. To Mutz and Futz, Brenda, Andy and Linda, Peggy and soon to be brother-in-law Cyril, I offer my sincerest thanks for your unshakeable faith in me and your willingness to endure with me the vicissitude of my endeavors. To my children, DeAnna and Kelly, I thank you for your innocent trust and for not imprinting on either our dogs or my picture during my frequent absences.
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on the development of immune responses to canine distemper virus in

Nara, P.L.: Effects of corticosteroids on the immune response. In:
(Eds. Powers and Bowens) Proceedings of the 2nd Equine Pharmacology
Symposium, 1979, pp 273-274.

effects of chloramphenicol on the immune responses to canine dis-
temper virus in beagle pups. J. Vet. Pharmacol. Therap. 5, 177-185,
1982.


FIELDS OF STUDY

Major Field: Comparative Pathobiology

Studies in Comparative Tumor Biology, Comparative Infectious Diseases
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5.6 Autoradiograph of an 0.5% agarose gel containing 125I-labeled serum lipoproteins (LPs) from fasted BALB/c mice. These were previously isolated by potassium bromide gradient ultracentrifugation and radio-iodinated as described in Materials and Methods. Lanes are in numerical order and represent the following LPs: Chylomicrons (origin) and very low density LPs (pre-alpha), lanes 1-3; intermediate density and low density LPs, lanes 5-9; and high density LPs (alpha), lanes 10-14 .......... 202
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Figure

5.7 Autoradiograph of the apolipoproteins from various $^{125}$I-labeled, delipidated lipoproteins isolated from BALB/c mouse thoracic duct lymph and serum by potassium bromide (KBr) gradient ultracentrifugation. Lane 1 represents the apolipoproteins from chylomicrons isolated from 1 KBr gradient. Lane 2 contains apolipoproteins from serum chylomicrons also isolated from 1 KBr gradient. Lane 3 contains apolipoproteins from high density lipoproteins isolated from the same gradient as the previous lane...

5.8 SDS-polyacrylamide gel of OIF transfer experiments utilizing a double-staining technique for the evaluation of apolipoprotein transfer(s) to thoracic duct chylomicrons (TDCs). Gel A (silver stained) contains the following: Lane 1, molecular weight markers; lane 2, TDCs only; lane 3, TDCs reisolated following an incubation with high density lipoproteins; lane 4, high density lipoproteins only; lane 5, chylomicrons only. Gel B is the same gel stained with Coomassie blue...

5.9 SDS-polyacrylamide gel autoradiograph of transferred $^{125}$I-labeled high density lipoprotein (HDLs)/apolipoprotein to thoracic duct chylomicrons (TDCs). Lane 1 represents fractions collected from the top of the control TDCs; lane 2 represents normal $^{125}$I-labeled HDLs; lane 3 represents TDCs reisolated from a potassium bromide gradient following the incubation of iodinated HDLs; lane 4 represents material taken from the fractions (d<1.006) of control iodinated HDLs from the gradient described for lane 1 and exposed to film twice as long as those required for lanes 1-3.

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HISTORICAL REVIEW AND INTRODUCTION

Both man and other animals are known to have a certain degree of nonspecific resistance to many viral infections (1). The mechanisms of this nonspecific host resistance are, however, incompletely understood. Part of this host resistance may be due to inhibitors that are not dependent upon prior immunization or induction by viral infection. These inhibitors often can be initially characterized by their carbohydrate, lipid, or protein composition and by their biological properties (2-5). Various anti-viral factors have been found in serum fractions (6,7), body fluids/secretions (7,8), and in tissue culture systems (6-9). A complete list of the various factors is presented in Table I-1.

Generally, all of these factors are considered to be nonimmune-activated or derived, with the possible exception of interferon. Of these factors listed, only two are reported to have an effect on viruses associated with the induction of neoplasia, i.e., murine oncornavirus-inactivating factor and the New Zealand Black factor. However, only the former is produced in vivo and thus retains the distinction of being the only naturally produced factor to have an effect on tumor-causing retroviruses.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Interferon</td>
<td>(2)</td>
</tr>
<tr>
<td>2. Myxovirus inhibitor</td>
<td>(1,3,4,5,6)</td>
</tr>
<tr>
<td>3. Togavirus inhibitor</td>
<td>(4,6)</td>
</tr>
<tr>
<td>4. Vaccinia virus inhibitor</td>
<td>(3,4,6)</td>
</tr>
<tr>
<td>5. Paramyxovirus inhibitor</td>
<td>(3,4,6)</td>
</tr>
<tr>
<td>6. Milk inhibitor</td>
<td>(7)</td>
</tr>
<tr>
<td>7. Cell produced viral inhibitor</td>
<td>(8,9,10)</td>
</tr>
<tr>
<td>8. Vesicular stomatitis virus inhibitor</td>
<td>(11,13)</td>
</tr>
<tr>
<td>9. Visna virus inhibitor</td>
<td>(12,13)</td>
</tr>
<tr>
<td>10. Influenza C virus inhibitor</td>
<td>(14)</td>
</tr>
<tr>
<td>11. Gibbon ape oncornavirus lytic factor</td>
<td>(15)</td>
</tr>
<tr>
<td>12. Murine oncornavirus inactivating factor</td>
<td>(16,17,18,19,20)</td>
</tr>
<tr>
<td>13. Type C New Zealand Black inhibitor</td>
<td>(21)</td>
</tr>
</tbody>
</table>
Table I.2 Characteristics of Various Viral Inhibitors

<table>
<thead>
<tr>
<th>Property</th>
<th>Viral Inhibitor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonspecific inhibition of viruses</td>
<td>(1,4,6)</td>
</tr>
<tr>
<td>Low molecular weight (&lt; = 2500)</td>
<td>(6)</td>
</tr>
<tr>
<td>Stable at 100°C</td>
<td>(4,7,10,13)</td>
</tr>
<tr>
<td>Stable in lipid solvents</td>
<td>(1,2,7)</td>
</tr>
<tr>
<td>Activity in cells of heterologous species</td>
<td>(2,3,4,5,6,7,8,9,12,13)</td>
</tr>
<tr>
<td>Induction of stable antiviral activity in cells</td>
<td>(1)</td>
</tr>
<tr>
<td>Produced spontaneously in absence of infection</td>
<td>(2,3,4,5,6,7,8,9,10,11,12,13)</td>
</tr>
<tr>
<td>Reversible inhibition of virus</td>
<td>(2,3,4,5,6,7,8,10)</td>
</tr>
</tbody>
</table>

*Number indicates the inhibitor listed in Table I.1
For a number of years the prevalent ecotropic murine C type oncornaviruses were thought to have been the causative agents of natural and experimentally induced murine leukemia (22-24). Further investigation revealed in the DNA of all mouse cells multiple proviral copies, some of which could be induced by various means to yield both eco- and xenotropic mouse leukemia virus (MuLV) isolates (25,26). More recently, further complexity became evident by the isolation of nondefective, amphotrophic viruses which were shown to be recombinants of eco- and xenotropic (MuX) variants (27,28). Based on phenomena in preleukemic states of natural disease in the high leukemia incidence AKR mouse strain, it was conjectured that it was the recombinant virus rather than the ecotropic virus that was responsible for induction of the disease process (28). The isolation of such a recombinant virus (HIX) in pure form from Moloney (M-) MuLV stocks allowed the demonstration that the recombinant virus was oncogenic by itself in the absence of detectable ecotropic virus (29). HIX virus-induced lymphomas contained ample amounts of only HIX virus which, even after cloning, was able to induce de novo lymphomas in mice, thus fulfilling the accepted postulates of causality (30). Analogous recombinant viruses were isolated from lymphomas of AKR mice; these were described as mink cell cytopathic agents (MCF) (28). From functional and structural points of view, HIX and MCF viruses are analogous, but they originated from different parental viruses (30,31). Although it has been demonstrated that HIX and MCF are "intragenic" recombinants, recently further viruses were isolated which were recombinants encompassing a substitution of whole
genes (30). The well-known defective recombinant subgroups of MuLV-derived oncogenic agents such as the various isolates of murine sarcoma virus (MSV) or the Friend spleen focus-forming virus (SFFV) represent further variations of oncogenic murine C-type agents. What is clear from the above discussions, is that extensive variation in the MuLV-derived group of viruses is quite common and that both defective and nondefective recombinant variants occur frequently. It is of interest that essentially all such oncogenic isolates were found to be recombinants in the general area of the envelope gene region of viral RNA. It has been demonstrated that HIX induces lymphoma, however the pure form of the virus is never isolated from animals that contain this serum factor (OIF), except in cases where overwhelming disease may interfere with the production of this factor (29). This appears to be the case when Balb/c mice are in their terminal stages of the disease and their liver parenchyma have been replaced in as much as 80% or more by neoplastic cells. This virally-induced lesion, in combination with the production of tremendous amounts of virus, leads to the isolation of HIX from serum, a condition never seen until the aforementioned embarrassments have been imposed upon the system. Some of the viruses that are isolated in these diseased animals, however, are found to have an ecotropic host range and later shown to be "genomically masked" (the HIX virus adopts the ecotropic envelope through recombination in situ). Thus, it appears that OIF may play a significant role in the natural selective pressures at work in the generation of virally induced lymphoma. Also to date, no circulating natural antibody to
xenotropic or recombinant viruses have been found within this species (32,33).

Normal mice contain murine leukemia virus (MuLV) proviral sequences in their DNA, and infectious xeno- and ecotropic virus can be induced from many mouse cells (25,34,35). These murine C-type RNA viruses are integrated as DNA copies into the chromosome of the mouse and can be passed by the germ cell to successive generations and are thus termed endogenous viruses. Extensive evidence has accumulated that the mouse is not immunologically tolerant to virus-coded envelope antigens or virus-structural antigens. Two types of immunologic responses to endogenous C-type viruses have been detected in normal mouse sera. Immunoglobulins of the IgM and IgG classes, as determined by radioimmunoprecipitation, with titers ranging between 1:10 and 1:50000, depending on the mouse strain (36), were found to bind to the virus envelope or virus-surface antigens of both ecotropic and xenotropic viruses (37,38) and have been previously described. These same sera, however, neutralize only the xenotropic viruses and have titers that range from 1:100 to 1:10,000 (39) and were found in all laboratory mouse strains tested at this time. The lack of correlation of neutralization with binding antibodies was unexplained and considered to be differences in the affinity of the immunoglobulins.

Levy et al. in 1975 (17) and Fischinger et al. in 1976 (16) published conclusive evidence that viral inactivation was not related to serum immunoglobulin and, as such, was described as a nonimmune serum factor. The factor prior to 1985 was characterized resistant
to 2-mercaptoethanol extraction, 40% ammonium sulfate precipitation, heating at 100°C for 10 minutes, immunoabsorption, multiple freeze/thaw cycles, lyophilization, pH ranges from 2-10 for 4 hours, perchloric acid, ether extraction (activity remains in the aqueous phase), pronases, protease K, trypsin, pepsin, DNase, RNase, alpha amylase, hyaluronidase, neuraminidase, and phospholipase A (16-19). The factor was sensitive to: chloroform/methanol (2:1) extraction, pH \( \leq 12 \) (17); multiple adsorption with normal and infected mouse and cat fibroblasts (S+L, FG-10, Gross-MuLV/3T3FL, FEF, MuX/FEF cells) (16); and rabbit anti-mouse HDL's (19). Preliminary studies indicated that the factor resides in the chylomicrons (CHYLO), very low density, and high density lipoproteins (19) (VLDL and HDL's, respectively). Also in 1982 it was demonstrated to transfer to human lipoproteins (20).

Due to the multiple interesting implications of this retroviral/lipoprotein interaction, the following major topics for research were considered and constituted the basis for the doctoral dissertation:

1. Due to the apparent species distribution of this factor, what is the distribution of the factor within all known Genera and subspecies of the mouse? Does it appear rapidly or gradually and can it be considered in terms of murine evolutionary phylogenetics?

2. Inactivation occurs in vitro with this factor, however the mechanism by which this occurs has not been demonstrated, nor has the in vivo protective aspects of the factor been considered.

3. Where is the factor synthesized, organ, cells, subcellular locations?
4. What is the developmental biology of the factor, such as levels in utero, following birth, and possible dietary effects influencing levels.

5. Biochemical characterization of the factor, isolation and purification.
REFERENCES


CHAPTER I

LIPOPROTEIN-ASSOCIATED ONCORNAVIRUS INACTIVATING FACTOR
IN THE GENUS Mus: EFFECTS ON MURINE LEUKEMIA
VIRUSES OF LABORATORY AND EXOTIC MICE

INTRODUCTION

Inactivation of xenotropic and polytropic recombinant murine leukemia virus (MuLV) in vitro by a nonimmunoglobulin, lipoprotein class-associated serum factor has been reported (1-5). This serum-derived oncornavirus inactivating factor (OIF) activity appeared to be associated with serum lipoproteins comprising the chylomicrons (CHYLOs), as well as very low density lipoproteins (VLDLs) and high density lipoproteins (HDLs) (4,5; Nara et al., unpublished observations). The nature of the association of OIF activity with lipoproteins has not been defined. Its quantity varied directly with the degree of lipemia, and under certain circumstances, could be detected in the VLDL fraction of mouse sera which were previously considered to be without activity (4,5). Passive transfer of OIF between lipoproteins of a high titer (B6C3)F, to the low OIF titer NSF/N mice has been reported (6). Although the mechanism of action of OIF is unknown, the very limited specificity of OIF for xenotropic and
recombinant MuLVs was of interest. The potency of this activity was remarkable in that OIF was present in higher titers in normal mouse sera than most neutralizing antibodies prepared against the gp70 of Friend (F-MuLV) (2).

Although the eco-, xeno- and amphotropic MuLVs, as well as their recombinants, have been extensively studied, much less is known of a family of MuLV-related sequences termed C-I (7). C-I viruses, considered to be xenotropic in nature, have been isolated from a number of mouse species, but never from the Mus M. musculus-derived laboratory mouse (8,9). Additionally, standard C-type MuLV-related viruses, termed C-II, and considered analogous to ecotropic MuLVs, have been induced from several exotic mouse species (10). It was of interest to determine whether these viruses would be susceptible to OIF, and whether a homologous OIF-type activity existed in other species to their own endogenous viruses.

Sera of other species (i.e., horse, cat, human) did not have OIF activity for the xenotropic or recombinant MuLVs (1,4). Furthermore, normal feline sera do not have an OIF for either their endogenous xenotropic virus RD114 or the partially exogenous ecotropic feline leukemia virus (4). In addition, rat serum is also negative for OIF. Therefore, it was of interest to determine phylogenetically whether related mouse species, semispecies, and subgenera contain OIF, and whether a gradual or sudden decrease in OIF activity was manifest. Finally, based on an unpublished report by Rowe (8) which indicated OIF to be under single dominant autosomal gene control, preliminary
simple genetic backcrosses were attempted between OIF-positive (+) and OIF-negative (-) mouse strains.
MATERIALS AND METHODS

Cells

Cell lines used in these experiments for the production of viruses included diploid feline embryo fibroblasts (FEF), mouse fibroblasts (3T3FL and SC-1) and the mink lung cell line (CCL-64). Other transformed cell-line derivatives used in neutralization assays were the sarcoma positive, leukemia negative (S+L-) mouse FG-10 cell line, and the S+L- cat (clone 81) cell line. All of the aforementioned cell lines were maintained in McCoy's 5A medium with 5 to 15% fetal calf serum as detailed elsewhere (11-14).

Viruses

The ecotropic MuLV (1869) used in these experiments was derived from Moloney-MuLV, biologically cloned by multiple-cycle, single-focus isolation, and end-point limiting dilution techniques (15). The Moloney MuLV polytropic recombinant MuLV (RM-M<sub>HIX</sub>) has also previously been described (16). Similarly, the following murine sarcoma virus pseudotypes have also been previously characterized: m<sub>1</sub>MSV simian sarcoma-associated virus (SSAV) (17), m<sub>1</sub>MSV BALB/c-derived murine xenotropic MuLV (B-MuX) (11), and m<sub>1</sub>MSV coated with endogenous cat RD114 helper virus (11).

Wild endogenous type C-I and C-II <i>cookii</i> MuLV were isolated by cocultivation of <i>M. cookii</i> spleen cells with NIH/3T3, SC-1 cells, and feline embryo fibroblasts (FEC). Isolation of the two viruses was accomplished by end-point limiting dilution techniques on non-mouse and mouse indicator cells, respectively, reverse transcriptase (RT)
analysis, and neutralization patterns. Type C-I (M720/FEC) replicated in FEC cells and was neutralized by antiserum to SSAV (9). M. cookii type C-II was sensitive to anti-Moloney sera and replicated only in mouse cells (unpublished data).

Type C-I replicated in feline embryo cells (M720/FEC) with neither focus formation nor focus induction in cells of several species, and had to be assayed for by RT methods (unpublished observations). Therefore, focus-forming pseudotypes were made to easily detect the sensitivity of these MuLVs to OIF.

Supernates from type C-I cookii-infected FEC cells were used to infect both clone 81 and FG-10 cells. The cat clone 81 cells, but not the FG-10 cells, exhibited a low number of large syncytia upon initial passage. Clarified supernates from these cells were incubated with mink lung cells (CCL-64) and produced numerous well-defined MSV-type foci within 5 days. These cells were used as the virus-producer cultures for the pseudotype m\textsuperscript{1}MSV (type C-I cookii) in these experiments.

Two strains of the other class of wild endogenous type C-II leukemogenic (leuk) and nonleukemogenic (nonleuk) MuLVs were derived from a lung cultures of the Asian mouse, M. cervicolor, after treatment with 5-bromodeoxyuridine and cocultivation with heterologous cells (10). One of these strains was leukemogenic in mice and the other was not. These two viruses, as well as type C-II cookii were propagated in NIH/3T3FL cells. The persistently infected cells were used in typical infectious cell-center assays at a total concentration of \(10^2\) to \(10^3\) cells per 60mm dish, containing various S+L- cells.
without distinct focus formation. However, when supernates of the infected FG-10 cells were used to inoculate either SC-1 or 3T3/FL cells, numerous distinct MSV pseudotype foci appeared. C-II *Cookii* always generated a lower titer of MSV virus than the *cervicolor* strain from S+L- indicator cells. The 3T3/FL cell line infected with the pseudotypes m<sub>1</sub> MSV (*cervicolor*-leuk), m<sub>1</sub> MSV (*cervicolor*-nonleuk), and m<sub>1</sub> MSV (*cookii* C-II) provided the viruses for the neutralization assays used in these experiments.

Source of sera

Mouse sera from 22 exotic species representing all 4 subgenera of the family *Muridae* and some backcrosses were examined. These animals were maintained in the exotic mouse colony at Litton Bionetics, Rockville, MD. All of these mice were maintained on a diet of Purina laboratory chows (Ralston-Purina Co., St. Louis, MO), supplemented weekly with 2 oz per cage of wild bird seed and wax moth larvae (2-3/mouse) as supplemental sources of protein. Nonfasted sera from twelve to twenty-four week old male and/or female mice representing each exotic species were collected. All blood was collected at the same time from all species following metofane inhalation anesthesia (Pitman-Moore, Inc., Washington Crossing, NJ) and exsanguination by cardiac puncture. The serum was allowed to clot, refrigerated overnight, and finally centrifuged. All sera were heat-inactivated and used in the assays the next day. Common laboratory mouse strains, i.e., BALB/c and C57BL/6, used in these and previous studies were obtained from the Animal Production Area at the NCI-Frederick Cancer Research Facility, Frederick, MD.
Virus inactivation of neutralization assays

Ecotropic Moloney MuLV (1869) was titrated in FG-10 mouse S+L-cells and the sarcoma virus pseudotypes $m_1$ MSV (RM-M\textsuperscript{HIX}), $m_1$ MSV (B-MuX), and $m_1$ MSV (SSAV) were titrated in mink lung cells (CCL-64). Wild-type C-I cookii MSV pseudotype was also quantitated in mink lung cells. The three exotic mouse derived C-II sarcoma pseudotypes ($m_1$ MSV cookii C-II), $m_1$ MSV (cerv-leuk), and $m_1$ MSV (cerv-nonleuk) were titrated in 3T3FL cells. Assays for mouse serum-inactivating factor were performed as previously described (4). Briefly, a dilution of heat-inactivated mouse serum was incubated with approximately 200 focus-forming or focus-inducing units of virus (FFU or FIU, respectively) for 1 hour at 25°C, and the resulting mixture was plated on appropriate indicator cells. The relationship in focus reduction by the serum at a given dilution can be expressed as the OIF inactivation units (O.U.) as related to the virus surviving fraction ($V_n/V_0$) (4), and calculated by the formula, O.U. = (0.5) (1/$V_n/V_0$) (1/dilution of serum). Levels of OIF <50 O.U./ml are considered nonsignificant. This value was based on nonspecific neutralization of both ecotropic and recombinant MuLVs with artificial lipoprotein preparations.

Electrophoresis of OIF

To determine the nature of virus inactivation factor found in various exotic mouse sera where serum volumes were of limiting volumes ($<1.0$ ml), sera could be separated into their constituent lipoprotein parts by agarose gel electrophoresis (18). Immunoglobulins were separated from these lipoproteins in 0.5% agarose gels according to the method of Paoletti et al. (19) and Garvey et al.
Serum 50 μl each) was loaded into duplicate 75 x 50 mm 0.5% agarose gels. Following the addition of a bromophenol blue stained albumin solution as a marker, the gels were run at 120 mA per gel in a 0.05 M barbital buffer bath (pH = 8.3) for approximately 2-1/2 hours, or until the albumin marker had moved 45 mm into the gel. One gel was fixed and stained with Oil Red O to localize lipoprotein, and served as the template for the native duplicate gel. Sections containing various classes of lipoproteins were excised from the duplicate gel and placed into dialysis bags. These were then placed back into a shallow trough of barbital buffer and electro-eluted at 120 mA for 1 hr. The polarity of the current was reversed at the end of the run to release any lipoprotein from the inner wall of the bag. The samples were then collected by Pasteur pipette and one small-volume wash of the dialysis bag with phosphate-buffered saline. This material was then thrice dialyzed against 1000 volumes phosphate-buffered saline lyophilized, reconstituted to original serum volume with PBS, filtered, and used in the neutralization assays.

**Genetic linkage studies**

A preliminary genetic study involving an OIF(+) mouse strain (BALB/c) and an OIF(-) mouse strain (M. spretus) was investigated. Originally a BALB/c male (OIF+) was crossed with an M. spretus OIF(-) female and an F1 female offspring, which were then backcrossed to either BALB/c or M. spretus to yield various backcrossed generations. Sera from these animals were collected and assayed for OIF activity.
RESULTS

Distribution of OIF in the genus Mus

Inactivating activity in the various sera was determined against the susceptible recombinant MuLV, RM-M\textsubscript{HIX}. In all cases tested, OIF-inactivated RM-M\textsubscript{HIX} or standard xenotropic MuLVs with equal efficiency. The genus Mus consists of four subgenera. The evolutionary oldest subgenus, Coelomys, represented in Table 1.1 by M. pahari, appeared to have no OIF. Pyromys, the next oldest subgenus, represented by M. platythrix, also exhibited no OIF. The remaining two subgenera, Nannomys and Mus, represent vigorous evolution in the family Muridae, predominantly based on the large number of species and overlapping distributions of these species (21). Nannomys, represented by M. minutoides, also had no OIF in their serum.

The subgenus, Mus, on the other hand, appears to be the only subgenus that contains species which have OIF+ sera. Generally, the Mus exotic species tested, such as M. caroli, M. spretus, M. cervicolor, and M. hortulanus, were found to contain insignificant levels of OIF in their serum. However, M. oookii, which is closely related to M. cervicolor based on 85% homology with labeled DNA probes (8), apparently had significant high titers of OIF at the level previously found in standard laboratory mice. M. musculus domesticus and M. musculus musculus both appear to contain a majority of subspecies that contain significant amounts of OIF. The highest levels (10,000 O.U./ml) were found in M. musculus musculus (Czech I) which
is comparable to levels detected in laboratory mice normally ranging between $10^3$-$10^4$ O.U./ml.

**Separation and recovery of OIF in electrophoretically separated lipoprotein fractions**

Originally, inactivating activity of OIF was mistaken for IgM neutralizing antibody (Lee et al., 1974). To separate out OIF from possible co-existing antibody, density gradient ultracentrifugation has been used to isolate the chylomicron (density <1.006 g/ml) and the HDL lipoprotein fractions containing OIF (density <1.021 g/ml) from serum antibodies which remain at higher specific densities in the gradient (4; Nara et al., unpublished observations). These gradients, however, require larger volumes of serum (4.0 ml/gradient) than were available from these exotic mouse colonies. Therefore a standard method of separating small volume samples was assessed to determine the efficiency of separation and recovery of active OIF.

A typical migration profile in Fig. 1.1 compared BALB/c serum, *M. cookii* serum, and a control mouse IgG/IgM mixture. The samples were stained with Oil Red O and Coomassie blue to determine the location of lipoproteins and proteins, respectively. In these gels, chylomicrons were found within 102 mm on the anodal side of the sample well, VLDL in the region from the pre-Beta ($\beta$) to alpha ($\alpha$), and HDL in the $\alpha$ region (approximately 30-40 mm from the origin). Immunoglobulins migrated toward the cathode in lane 5 (Fig. 1.1). Goat anti-Friend MuLV gp70 back-migrated to the same gamma ($\gamma$) position, as did the mouse Ig preparation. The sections of gels were
cut, electroeluted, and tested for OIF. Because of possible immunoglobulin overlap in the α-region, only fasted sera was used so as to avoid OIF activity associated with dietary-induced chylomicrons found in this region. Under these conditions, the bulk of the OIF activity (60%) is found associated in the alpha region, and assures the sample from immunoglobulin contamination. Recovery of OIF activity as the sum of the eluted fractions was very reproducible and ranged between 85% and 100% of the original serum sample. Eluted serum fractions were compared in terms of OIF units for recombinant and ecotropic MuLV as found in Table 1.2. BALB/c mouse serum had highest level of OIF in the HDL fraction, and the next highest in the chylomicron VLDL (pre-β) fraction.

The same proportional distribution of OIF was found in the lipoprotein fractions of the M. cookii serum. Neither serum had any activity in the region where immunoglobulins migrated. Also, neither serum contained any inactivating activity against the ecotropic M-MuLV. In contrast, the hyperimmune goat IgG migrated to the gamma region where all the activity of the antibody was detected. Neither albumin nor mouse IgG had any OIF activity. Based on the above results, it appeared that the inactivating activity in the M. cookii serum was true OIF, because it was lipoprotein associated, was not immunoglobulin, and retained a specificity for recombinant or xenotropic MuLVs without inactivation of the ecotropic MuLV prototype.

Endogenous MuLVs derived from exotic mice

A limited number of MuLVs have been induced from exotic mouse species (7,8,10,23). For example, a class of MuLVs termed C-I, which
have never been isolated from laboratory mice, can readily be isolated from *M. cookii cervicolor*, *caroli pahari*, *shorteridgei*, and *dunni*. The only previously known viruses related to C-I, as defined by hybridization and antigenic reactivity, are the two primate viruses, known as SSAV and gibbon ape leukemia virus (GaLV). Another class of MuLV, termed C-II MuLVs, were isolated from *M. cookii* or *M. cervicolor*. These viruses replicate (positive RT activity) in mouse cells and appear, by immunological criteria, to be closely related to the typical laboratory MuLVs.

Host range determination of these viruses was assessed by the formation of MSV pseudotypes as well as by the determination of FIU in various S+L- cells (Table 1.3). C-I virus produced syncytial foci in cat S+L- cells, and these supernates contained the m<sub>1</sub>MSV (*M. cookii* C-I) pseudotype. This virus pseudotype was also able to enter mouse cells as determined by efficient formation of MSV foci in normal mouse cell lines, including 3T3FL cells, if appropriate ecotropic MuLV was added as helper. Permissive entry of pseudotype without replication has been described previously (12). C-I virus did replicate readily in both mink and cat cells. The C-I related SSAV was similar in that it replicated well in heterologous feline and rabbit cells. However, m<sub>1</sub>MSV (SSAV) was not able to enter either mouse 3T3FL or SC-1 cells.

The three C-II isolates had properties compatible with mouse ecotropic MuLVs. Although focus induction by these C-II viruses was not measurable in standard mouse S+L- FG-10 cells, ample (<10<sup>4</sup> FFU/ml) m<sub>1</sub>MSV (C-II pseudotypes) were produced in all cases. These
pseudotypes entered into mouse cells, but not into cat or mink cells. The C-II viruses also replicated adequately in standard mouse cells but did not grow in non-mouse cell lines.

These exotic mouse-derived pseudotypes were next assayed to determine whether they were susceptible to standard laboratory mouse OIF or to goat antibodies having high titers of neutralizing antibodies including the group and interspecies specific types. For example, hyperimmune goat anti-Friend MuLV gp70 had titers as high against RM-HIX and B-MUX as it did to F-MuLV. It also has low titer reactivity against FeLV (24). As seen in Table 1.4, when comparable titers of either BALB/c (not shown) or C57BL/6 serum were used, the standard recombinant and xenotropic MuLV pseudotypes were readily inactivated. The other C-I or C-II pseudotypes were not affected. The hyperimmune MuLV group-reactive serum had high titers, as expected, against the standard MuLV pseudotypes. Surprisingly, the m^1MSV (C-II cookii) was exceedingly susceptible to this antibody. In contrast, the C-I m^1MSV pseudotype from M. cookii, as well as the two C-II cervicolor m^1MSV pseudotypes, were significantly less well neutralized, and finally, the standard m^1MSV(SSAV) which was not affected at all.

Do sera of exotic mice have an OIF for homologous endogenous MuLVs?

Since laboratory mice had OIF which was specific for xenotropic or recombinant viruses of its own subspecies, it is possible that other species-specific OIF system(s) exist. To determine if this were the case, sera of exotic species were tested against available homologous and heterologous viruses. Mus spretus was of interest
because it could be crossbred with laboratory mice. Sera of two varieties of *M. cervicolor* were available. Table 1.5 describes the existing available MuLV pseudotypes tested for inactivation by the exotic mouse sera. Both the *M. cookii* and *M. cervicolor* systems contain matching homologous endogenous virus with the serum of the subspecies being tested. Apparently none of the exotic mouse sera had any OIF activity for any of the exotic MuLVs tested (Table 1.5).

In contrast, the *M. cervicolor cervicolor* serum did inactivate the non-leukemogenic variant of the C-II *cervicolor* MuLV at a high titer. To determine whether this was an example of the presence of true OIF or whether antibody was present, electrophoresis and electro-elution of the serum sample was performed using fractions as indicated in Fig. 1.1. All activity was recovered in the gamma region (immunoglobulin containing), and was specific for the non-leukemogenic isolate. No inactivating activity was found in any lipoprotein-containing gel regions.

**Inheritance of OIF in backcrosses of BALB/c and M. spretus**

As mentioned, previous studies by Rowe utilizing NSF as an OIF(-) strain suggested that the factor is determined by a single dominant autosomal gene. However, these experiments are difficult to interpret because the NIH Swiss mice (which are related to NSF) were also reputed to be OIF(-), yet OIF could be detected in the VLDL lipoprotein fraction of serum of these mice following gentle centrifugal separation (4). Accordingly, a now documented OIF negative strain, *M. spretus*, which can crossbreed with BALB/c mice, has been bred to determine the inheritance of OIF in backcrossed mice. Four
backcrosses, N2 to N5, of the F₁ (BALB/c x M. spretus) to BALB/c were tested (Table 1.6). All mice tested were OIF(+) in all generations, however, the titers were quantitatively about one-third the normal BALB/c value. The titers of OIF were not substantially higher in N5 than in N2 animals. Only one N4 mouse had a titer close to a control mouse, i.e., 850 OIF units, compared to a fasting value of 1000 O.U. in a normal BALB/c mouse. Due to the small litter size in the original parental cross (2 females, 2 males), the F₁ hybrids were not sacrificed for OIF determinations.

Backcrosses to M. spretus were unsuccessful beyond N2. However, of the four N2's tested, three of the four were negative, and one was positive for OIF. The level of OIF in this mouse was compatible with the N2 level backcross to BALB/c mice.
DISCUSSION

The phylogenetic distribution of OIF was found to be limited to the genus Mus and to the semispecies, M. m. musculus and M. musculus domesticus, where it appeared at high titer. Other mouse subgenera, species, and subspecies had essentially no detectable OIF. The only exception was M. musculus cookii, which had true lipoprotein-associated OIF at a titer comparable to laboratory mice. Historically, most of the laboratory mouse strains evaluated for OIF are known to have originated from domesticated mice of North European and North American pet dealers and mouse fanciers. These mice were a mixture of locally caught North European (presumed M. m. domesticus origin) mice, subsequently carried across the Atlantic to North America, and imported "fancy" Chinese and Japanese mice (25). Bishop et al. (26) has shown recently that eight common laboratory strains, all known to have OIF, should be considered genetic hybrids between the two major semispecies (M. m. domesticus and M. m. musculus).

Whether the presence of OIF depends on pre-existing endogenous retroviral-related sequences can be considered. Clearly, no correlation exists between the presence of endogenous ecotropic-related type C viruses which are not susceptible to OIF and the presence of OIF in a given laboratory mouse strain (27,28). However, a stronger correlation exists with the presence of endogenous polytropic and/or xenotropic retroviral-related sequences and the presence of OIF. All common laboratory mouse strains contain OIF, and also have xenotropic and/or polytropic endogenous MuLV-related sequences in
their genomes (29). In contrast where studied, xenotropic and/or polytropic sequences are generally missing in OIF(-) strains of exotic mice such as M. spretus, M. pahari, M. cervicolor, and M. caroli, and present in OIF(+) strains such as M. m. castaneus, M. molossinus and M. poschiavinus (27,30). The only known exception is the M. m. cookii which is OIF(+), and yet apparently is negative for xenotropic MuLV sequences (27). DNA probes constructed from the C-I ecotropic virus isolated from M. m. cookii, however, do hybridize with all M. musculus inbred strains and M. poschiavinus (23). It is still possible that M. m. cookii contains xenotropic sequences that are not detectable with the hybridization probes previously used. Also, due to the limited number of species tested, and the use of different xenotropic-specific probes, further comparative studies using identical probes will be necessary to substantiate this initial observation. Finally, the very high degree of susceptibility of the C-II isolate of M. cookii but not other C-II viruses, to group-specific neutralizing anti-Friend MuLV gp70 suggests a degree of relationship between this strain and the other OIF(+) strains of mice.

Several different classes of endogenous retroviruses have been isolated from Asian species of Mus (8). On the basis of biological and biochemical criteria, two subclasses of type C viruses have been identified. Type C-I wild endogenous retroviruses isolated from M. cervicolor and M. cookii are represented in this study by type C-I M. cookii. This subclass of wild type C-I MuLVs apparently could enter into normal mouse cells, but only replicated in non-murine cell
lines. The C-I class of MuLVs have been isolated from all Mus species tested, with Mus m. musculus being the only exception (23). Beneviste et al. (10) considered type C-I viruses to be present in all members of the genus Mus, more evolutionarily conserved, and appeared to be the probable progenitor of the non-endogenous primate virus. This class of endogenous MuLV is closely related to SSAV or GaLV type C viruses found in primates based on nucleic acid hybridizations, competition of major structural p30 in a wooly monkey-gibbon ape group-specific radioimmunoassay, and cross-reactivities with reverse transcriptase and p30 (7). OIF from unrelated, related, and homologous Mus species failed to inactivate this C-I cookii virus. Also, group and interspecies-specific neutralization anti-Friend MuLV gp70 antisera were found to be significantly less active toward this virus than the standard xenotropic MuLVs. It is interesting to note that standard xenotropic MuLV (OIF sensitive) isolated from Mus musculus can infect M. cervicolor cells (1). However, these newer wild type C-I (xenotropic) and C-II (ecotropic-like cervicolar) isolates cannot. This may indicate an intraspecies envelope determinant that is associated with interspecies cellular recognition and OIF sensitivity. Thus, it appears that both C-I cookii as well as C-I cervicolar MuLVs are not sensitive to OIF, and may represent further biological evidence for a relationship between some type C-I MuLVs and the non-endogenous retrovirus of primates.

Type C-II MuLV, represented by C-II cookii and cervicolar, resembled N-ecotropic MuLVs from M. musculus. The viruses do not compete in the SSAV-C-I MuLV group-specific radioimmunoassay (8).
The antigenic determinants of the reverse transcriptase and p30 protein of the C-II cervicolor virus showed weak cross-reactivities with the homologous proteins of laboratory strain MuLVs, and the RNA genome of C-II cervicolor was partially homologous to endogenous type C viruses isolated from M. musculus cells. Of the numerous indicator cells tested to date, the C-II cervicolor virus replicated well only in cells derived from M. musculus. The available type C-II MuLVs were neutralized by anti-Friend MuLV gp70, with type C-II cookii being the most sensitive. OIF from all mouse strains failed to neutralize these MuLVs, however, type C-II cervicolor (non-leuk) had significant levels of antibody in its own serum for its own MuLV. It appeared from the OIF-inactivation profiles that type C-II MuLVs were generally ecotropic-like and behave as do other previously characterized ecotropic MuLVs in the uniform lack of inactivation by OIF (24).

When the sera of exotic mice were assessed for the presence of OIF to inactivate their own induced endogenous viruses, no OIF was detected. However, it is possible that the appropriate endogenous MuLVs from these species contain OIF-sensitive viruses that have not yet been isolated. It is remarkable that the M. cookii serum does have a high titer OIF for an apparently less related xenotropic MuLV but could not inactivate its own C-I or -II homologous isolates.

The inheritance pattern seen in this preliminary study is inconclusive and will require further investigation. The data, however, do indicate an easily measurable heritable trait and may
represent the emergence of an OIF resistance gene. More specifically, a resistance gene may have emerged during the evolutionary divergence of *M. spretus* from *M. musculus* and perhaps independently in *M. cookii*. This observation should provide the basis for future studies to identify the chromosomal location of this locus and its relationship to endogenous xenotropic sequences. Further studies should include the OIF activity of the N₁ generation along with approximately 50 to 100 backcross progeny from the N₁ × *spretus* cross.

The re-isolation of active OIF in electrophoretic fractions of mouse serum lipoproteins, including HDLs, allows for further purification and the determination of the mechanism of action of OIF. Specific protocols have recently been devised which allow for dietary host manipulation so that OIF levels increase by more than thirtyfold (unpublished observations). The availability of high titer purified OIF should be helpful toward the elucidation of the nature of the specificity and the efficiency of this unique viral inactivation process. Hopefully, the definition of this mechanism will allow for an extension of the use of this process to other retroviruses.
SUMMARY

High titers of oncornavirus-inactivating factor (OIF) were previously found in sera of laboratory mice. OIF is highly active against mouse xenotropic and polytropic envelope recombinant murine leukemia viruses (MuLVs), but not against ecotropic MuLVs. Of the twenty different mouse species or subspecies currently tested that represent four subgenera, no OIF was found in the three subgenera more distant to the laboratory mouse. In the subgenus Mus, seven of the eight most distant species had no OIF, whereas all the ancestral species and subspecies of the laboratory mouse (M. musculus musculus, M. musculus domesticus), including a more distant member (M. cookii) had ample titers of OIF. A new separation technique was devised so that potential virus-neutralizing immunoglobulins could be separated by electrophoresis from OIF in small volume serum samples. Active OIF was recovered from serum high-density lipoprotein (HDL), very low density lipoprotein (VLDL), as well as from chylomicron (CHYLO) fractions. Murine sarcoma virus (MSV) pseudotypes were made with several available exotic MuLV types. These pseudotype MuLVs were not susceptible to standard OIF preparations. The sera of exotic mice also had no factor analogous to OIF which would inactivate their own homologous or heterologous exotic MuLVs. In a limited backcross study using BALB/c and M. spretus, OIF was present in progeny from all N2-N5 backcrosses to BALB/c mice, but in only one of four mice of an N2 backcross to M. spretus. It appears that, with one exception,
OIF activity is limited to two subspecies of *M. musculus*, and may be correlated with the presence of endogenous xenotropic MuLVs.
Table 1.1 Presence of Oncornavirus-Inactivating Factor in Selected Species of the Mouse Family Groups Taxonomically (Foster et al., 1981)

<table>
<thead>
<tr>
<th>Genus/Subgenera of the Family Muridae</th>
<th>OIF Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coelomys*</td>
<td>Mus pahari</td>
</tr>
<tr>
<td></td>
<td>&lt;10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyromys*</td>
<td>Mus platythrix</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Nannomys*</td>
<td>Mus minutoides</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Mus cervicolor popaeus</td>
</tr>
<tr>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Mus cervicolor cervicolor</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>M. musculus caroli</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>M. musculus cookii</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
</tr>
<tr>
<td></td>
<td>M. hortulanus (Pancevo)</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>M. hortulanus (Halbtum)</td>
</tr>
<tr>
<td></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>M. spretus (Spain)</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>M. spretus (Morocco)</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>M. musculus domesticus (centerville light)</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>(poschiavinus)</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>(brevirostiris)</td>
</tr>
<tr>
<td></td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>M. musculus castaneus</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
</tr>
<tr>
<td></td>
<td>M. musculus musculus (Czech I)</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
</tr>
<tr>
<td></td>
<td>M. musculus musculus (Czech II)</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>M. musculus musculus (vejrumbro)</td>
</tr>
<tr>
<td></td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>M. musculus molossinus</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are represented as OIF units of which less than 50 O.U./ml are considered negative.
Table 1.2 Electrophoretic Separation of OIF.

<table>
<thead>
<tr>
<th>Sample Descriptiona</th>
<th>Sample Location</th>
<th>OIF Units</th>
<th>RM-HIX</th>
<th>E-MuLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c serum</td>
<td>γ</td>
<td>&lt;10(^b)</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>origin-β</td>
<td>210</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-β</td>
<td>100</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>600</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>M. cookii serum</td>
<td>γ</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>origin-β</td>
<td>120</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-β</td>
<td>40</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>550</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>Goat anti-gp70 serum</td>
<td>γ</td>
<td>300</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>origin-β</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-β</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Approximately 1,000 O.U. were loaded in the gel for both BALB/c and M. cookii serum.

\(^b\)Represents the number of OIF units calculated by the formula: O.U. = (0.5) (1/V_n/V_o) (1/dilution) with levels less than (<) 10 units to be considered immeasurable.
Table 1.3 Species Tropism of Endogenous MuLVs Derived from Exotic Mouse Species

<table>
<thead>
<tr>
<th>MuLV Class and Origin</th>
<th>Laboratory Mouse Cells</th>
<th></th>
<th></th>
<th>Non-Mouse Cells: Cat, Mink, Rabbit</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Repli-</td>
<td>cation</td>
<td></td>
<td>Entry</td>
<td>Repli-</td>
<td>cation</td>
</tr>
<tr>
<td>C-I MuLV:</td>
<td></td>
<td></td>
<td></td>
<td>from M. cookii</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C-I-related MuLV:</td>
<td></td>
<td></td>
<td></td>
<td>SSAV from primates</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-II MuLV:</td>
<td></td>
<td></td>
<td></td>
<td>M. cookii</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C-II MuLV:</td>
<td></td>
<td></td>
<td></td>
<td>M. cervicolor, leukemogenic or non-leukemogenic strains</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Entry is measured as detection of MSV foci in the cells of a species in the presence of exogenously added helper virus which grows well in the species, e.g., FeLV for cat cells.

Replication was measured as induction of reverse transcriptase activity and/or detection of focus inducing units in S+L- cells of various species.
Table 1.4 Inactivation of MuLVs from Exotic Mice by OIF and Hyperimmune Anti-Friend MuLV gp70 Serum

<table>
<thead>
<tr>
<th>MSV Pseudotyped by Assorted MuLV Types</th>
<th>Serum Source (OIF Units)</th>
<th>Mouse C57BL</th>
<th>Goat Hyperimmune anti-Friend MuLV gp70</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5,000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62,500</td>
</tr>
<tr>
<td>m&lt;sub&gt;1&lt;/sub&gt;MSV (RM-M&lt;sub&gt;HIK&lt;/sub&gt;)</td>
<td></td>
<td>5,300</td>
<td>50,000</td>
</tr>
<tr>
<td>m&lt;sub&gt;1&lt;/sub&gt;MSV (B-MuX)</td>
<td></td>
<td>&lt;10</td>
<td>1,400</td>
</tr>
<tr>
<td>m&lt;sub&gt;1&lt;/sub&gt;MSV (C-I cookii)</td>
<td></td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>m&lt;sub&gt;1&lt;/sub&gt;MSV (SSAV)</td>
<td></td>
<td>30</td>
<td>150,000</td>
</tr>
<tr>
<td>m&lt;sub&gt;1&lt;/sub&gt;MSV (C-II cookii)</td>
<td></td>
<td>20</td>
<td>4,540</td>
</tr>
<tr>
<td>m&lt;sub&gt;1&lt;/sub&gt;MSV (C-II cerv./n.l.)</td>
<td></td>
<td>&lt;10</td>
<td>1,900</td>
</tr>
<tr>
<td>m&lt;sub&gt;1&lt;/sub&gt;MSV (C-II cerv./l)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Represents the number of OIF units calculated by the formula: O.U. = (0.5) (1/V<sub>n</sub>/V<sub>0</sub>) (1/dilution) with levels less than (<) 10 units to be considered immeasurable.
Table 1.5 Exotic Mouse Sera do not have an OIF-Type Activity for Several Exotic MuLVs Tested.

<table>
<thead>
<tr>
<th>MSV Pseudotyped by MuLVs</th>
<th>M. cookii</th>
<th>M. cervicolor</th>
<th>M. cervicolor popaeus</th>
<th>M. spretus</th>
</tr>
</thead>
<tbody>
<tr>
<td>m₄ MSV (RM-M&lt;sub&gt;HIX&lt;/sub&gt;)</td>
<td>5,000</td>
<td>20</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>m₄ MSV (B-MuX)</td>
<td>3,000</td>
<td>&lt;10</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>m₄ MSV (C-I cookii)</td>
<td>20</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>m₄ MSV (C-II cookii)</td>
<td>20</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>m₄ MSV (C-II cerv./non-leuk)</td>
<td>20</td>
<td>5,000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>m₄ MSV (C-II cerv./leuk)</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>m₄ MSV (SSAV)</td>
<td>20</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

<sup>a</sup>This activity was shown to be immunoglobulin-mediated, see text for details.
Table 1.6 The Inheritance Pattern of OIF in Crosses of BALB/c and _spretus_ Mice.

<table>
<thead>
<tr>
<th>Backcross Description</th>
<th>OIF Units</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F_1</em> (BALB/c-spretus) female x BALB/c male</td>
<td></td>
</tr>
<tr>
<td>( N_2 \times \text{BALB/c} )</td>
<td>male 150(^a)</td>
</tr>
<tr>
<td></td>
<td>female 267</td>
</tr>
<tr>
<td></td>
<td>female 245</td>
</tr>
<tr>
<td>( N_3 \times \text{BALB/c} )</td>
<td>female 240</td>
</tr>
<tr>
<td></td>
<td>male 300</td>
</tr>
<tr>
<td>( N_4 \times \text{BALB/c} )</td>
<td>female 330</td>
</tr>
<tr>
<td></td>
<td>male 850</td>
</tr>
<tr>
<td>( N_5 \times \text{BALB/c} )</td>
<td>male 250</td>
</tr>
<tr>
<td></td>
<td>female 250</td>
</tr>
<tr>
<td><em>F_1</em> (BALB/c-spretus) female x <em>spretus</em> male</td>
<td></td>
</tr>
<tr>
<td>( N_2 \times \text{spretus} )</td>
<td>female 50</td>
</tr>
<tr>
<td></td>
<td>female 50</td>
</tr>
<tr>
<td></td>
<td>female 50</td>
</tr>
<tr>
<td></td>
<td>female 167</td>
</tr>
</tbody>
</table>

\(^a\)Represents the number of OIF units calculated by the formula: 
\[
\text{O.U.} = \left(0.5\right) \left(\frac{1/V_n}{V_0}\right) \left(1/\text{dilution}\right)
\]
with levels less than (<) 10 units to be considered immeasurable.
Fig. 1.1 Separation of various mouse sera by 0.5% agarose gel electrophoresis. Duplicate gels were used in electrophoresis/electro-elution determinations for OIF activity. Well 1: 25 μl of whole BALB/c serum stained with Oil Red O. Well 2: Coomassie stain of same preparation. Well 3: 25 μl of whole M. cookii serum stained with Oil Red O. Well 4: Coomassie stain of same preparation. Well 5: 50 μl of IgG/IgM mouse immunoglobulin (Coomassie stain).
Table 1.5 Exotic Mouse Sera do not have an OIF-Type Activity for Several Exotic MuLVs Tested.

<table>
<thead>
<tr>
<th>Exotic Mouse Sera (OIF Units)</th>
<th>MSV Pseudotyped by MuLVs</th>
<th>M. cookii</th>
<th>M. cervicolor</th>
<th>M. cervicolor popaeus</th>
<th>M. spretus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m^1 MSV (RM-M\text{HIX})</td>
<td>5,000</td>
<td>20</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>m^1 MSV (B-MuX)</td>
<td>3,000</td>
<td>&lt;10</td>
<td>40</td>
<td>20</td>
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\textsuperscript{a}This activity was shown to be immunoglobulin-mediated, see text for details.
REFERENCES


CHAPTER II

THE EFFECT OF MURINE LIPOPROTEIN-ASSOCIATED ONCORNAVIRUS-INACTIVATING FACTOR ON INFECTIOUS VIRIONS, THEIR ADSORPTION AND PENETRATION

INTRODUCTION

Adsorption and penetration of retroviruses into host cells depends on specific interaction between the viral envelope glycoprotein and a cell surface receptor (1). This complex mechanism may be interrupted by a number of non-specific avenues (2), however, specific endogenous host defense mechanisms are limited primarily to the production of neutralizing antibody (3). Briefly, neutralizing antibody can; (1) bind to specific glycoproteins of the virions thus blocking initial adsorption; (2) bind to previously bound virions either eluting these particles, fixing them onto the membrane, initiating virolysis by fixing complement, and/or disturbing activation of various host cell membrane enzymes required for penetration; and finally; (3) bind to attached virions and promote inappropriate viral phagocytic/endocytic mechanism leading to abortive uncoating and processing of the viral genome (3). Specific information regarding the effects of neutralizing antibody on retrovirus adsorption and
penetration is noticeably lacking. Currently, only immunoglobulin containing serum and body fluids are known to affect viruses in such a diverse manner. However, numerous other non-immune antiviral serum factors have been described for both DNA and RNA viruses (4,5) and are found to effect virions at various stages of their infection/replication cycle. Only one, however, is produced endogenously in the mouse, has specific inactivating capacity for leukemogenic xenotropic and some polytropic recombinant retroviruses, and is found in titers higher than any reported xenotropic MuLV neutralizing antibody (6,7). This serum factor is characterized as a soluble non-immunoglobulin, lipoprotein class-associated, noncomplement-associated, heat/pH-stable constituent of all laboratory mouse strains derived from Mus musculus musculus and M. m. domesticus stocks. These reported lipoprotein classes include chylomicrons (CHYLOs), very low density lipoproteins (VLDLs), and high density lipoproteins (HDLs).

Other characteristics of the factor include a dependence of apolipoproteins in association with its natural lipid membrane for maximal activity; however, resultant reduction in activity is noted as these lipids and proteins are disassociated by various reagents (8,9). The term lipoprotein-associated oncornavirus-inactivating factor (OIF) has been previously applied to this factor (9) and for this study is synonymous with CHYLO/VLDLs and HDLs. Isolated apolipoproteins from these fractions have been reported to be transferable to human CHYLOs, low density lipoproteins (LDLs), and ordered lipid vesicles (10). Finally, the factor is reported only
within the subgenus: Mus, species: musculus, domesticus, and cookii in the mouse family (Nara et al. unpublished observations), with a large number of other unrelated species being tested (human, rat, feline, hamster, horse and chimpanzee) (9). It has been suggested in some of these reports that the factor may interact with the envelope of the virus. Prior to this report, however, no data on the mechanism of virus inactivation has been advanced to explain the nature or outcome of the virus-lipoprotein interactions. Thus, it was the intention of this study to begin characterization of this viral inactivation by asking the following questions: 1) does OIF bind to the virion, if so, is the binding specific (i.e., only binds to those classes of MuLVs that lead to inactivation)? 2) does OIF cause virolysis, if so, by itself or in association with cells? 3) what is the effect of OIF on virion adsorption/penetration of the host cell as compared to MuLV-specific neutralizing antibody? and 4) substantiate the lipoprotein classes reported to contain OIF and their activity in the aforementioned mechanisms.
MATERIALS AND METHODS

Cells and viruses

Mouse 3T3FL cells, one of their sarcoma-positive leukemia-negative (S+L-) cloned sub-lines, FG-10 and feline embryo fibroblasts have already been completely described (11,12). The ecotropic MuLV (1869) used in these experiments and grown in 3T3FL cells were derived from Moloney MuLV cloned by single focus isolation and endpoint limiting dilution techniques as described previously (12). The polytropic recombinant MuLV (HIX), produced in feline embryo fibroblasts (FEF), were derived from the infection of 3T3FL cells with Molony MuLV and isolated as described earlier (12). The mouse (S+L-) fibroblast cell lines (FG-10) were used for all inactivation and binding studies.

Animals

Inbred mature male and female BALB/c mice (obtained from the Animal Production Facility, Fredrick Cancer Research Facility, Fredrick, MD) provided the serum source for these experiments. The animals were maintained on Purina Mouse Chow and water ad libitum. Prior to exsanguination, all mice were fasted overnight. Of these, some animals were given olive oil via stomach tube 90 minutes prior to exsanguination while other animals were bled after the fasting period. All animals were anesthetized and blood collected following jugular transection. Blood from cat, rat, goat, and rabbit were collected as just described.
Blood samples and lipoprotein isolation

The blood samples were collected and pooled into 30 ml glass conical centrifuge tubes and allowed to clot at room temperature. Serum was separated, heat-inactivated and prepared for lipoprotein isolation as described by Redgrave et al. (13). Briefly, a 4 ml aliquot of serum was adjusted to $d=1.21$ by the addition of solid potassium bromide salt ($KBr$, $0.325$ g/ml of serum) in SW-41 centrifuge tubes. Continuous gradients were formed at ambient temperature with the use of a Haake Buchler Autodensiflow II (Buchler Instruments, Searle Analytical Inc., Fort Lee, NJ) gradient maker using KBr salt solutions of $d=1.063$ and 1.019 at a speed of 1.0 ml/min. Finally, the tubes were topped off with 2.0-3.0 ml of $d=1.006$ KBr solution containing 1.0 mM EDTA and 1% penicillin/neomycin/streptomycin. The samples were then centrifuged at 36,000 rpm for 18 hours at $15^\circ$C in a Beckman LS Model 350 ultracentrifuge and fractionated into 0.5 ml fractions with the aid of a Gilsen micro-fraction collector. The fractions were then checked for purity by agarose gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and chemical composition analysis; phospholipid, protein, triglyceride, total cholesterol and free cholesterol (1000D microassay, Litton Bionetics, Rockville, MD), with fractions containing the major lipoprotein classes of known density classes being pooled and dialyzed in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA; exclusion limit ca. 3,500) for 3X 24 hours at $4^\circ$C in phosphate-buffered saline.
Labeling and purification of virus for binding studies

Both HIX (recombinant MuLV) and 1869 (ecotropic MuLV) used in these experiments were metabolically labeled from infected cell cultures. $^3$H-leucine or $^{35}$S-methionine/cystine labeled MuLVs were prepared for RNA labeling by incubating fully infected 80-90% confluent T150 flasks with complete medium containing 200 μCi of $^3$H-uridine (specific activity 20 Ci/m mole).

Virions used for the cellular binding studies required a more energetic label and thus were incubated with a mixture of 2.5 mCi of $^{35}$S-methionine and 2.5 mCi of $^{35}$S-cystine (New England Nuclear, Boston, MA) for 12 hours at 37°C and 5% CO₂. The medium was pooled from 10 flasks and clarified at 9,000 rpm for 20 minutes at 15°C in a RC2-B Beckman centrifuge. The supernate from these runs were then pelleted in SW 27 tubes containing a 5.0 ml glycerol cushion and centrifuged at 25,000 rpm for 90 minutes at 15°C in a Beckman Model L350 ultracentrifuge. The pelleted $^{35}$S-labeled virus used in the adsorption/penetration studies were rinsed in cold complete McCoy’s medium one time and used immediately. The pelleted $^3$H-MuLV was rinsed in 1X phosphate-buffered saline (PBS) and banded on a continuous 15-65% (for HIX), 15-55% (for 1869) sucrose gradient.

Concentrated, purified virus was dialyzed overnight against 1X PBS. Aliquots containing 5,000 cpm/0.5 ml were then frozen, stored at -80°C and used within 2 weeks. All labeled viruses were examined for purity by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Specific biological activity of the $^3$H-labeled HIX and 1869 MuLVs were 0.05 plaque-forming units/counts.
per minute (PFU/cpm) and 0.10 PFU/cpm, respectively, after freezing. The specific activity of the $^{35}$S-labeled MuLV was determined to be 0.1 PFU/cpm for HIX and 0.15 PFU/cpm for 1869.

Binding and virolysis studies

The following studies were performed to determine if binding and/or virolysis are characteristics of OIF on ecotropic and xenotropic recombinant MuLVs. $^3$H-labeled 1869 (2,500 cpm/0.5 ml) and HIX (5,000 cpm/0.5 ml), approximately 250 FFU/virus were incubated with purified lipoproteins from mouse, rat, rabbit, goat and cat at varying concentrations: 0.1, 0.5, 1.0, 2.5, 10.0, 50.0, and 100.0 µg (apolipoprotein concentration). The lipoprotein classes included were: CHYLO/VLDLs, LDLs, or HDLs. Hyperimmune heat-inactivated goat Friend anti-gp70 (anti-gp70) (14) was used as a positive control and served to indicate the sensitivity of the assay. Also, various combinations of CHYLO/VLDL and anti-gp70 antibody were used with and without pronase (50 µg total concentration)-treated virus to determine the role envelope proteins (i.e., gp70) in the binding process. These incubations were carried out at 37°C in a shaker water bath for 30 minutes. Positive controls for the determination of virolysis included both detergent (NP40)-treated virus preparations or the addition of complement (low-TOX rabbit complement, Cedar Lane Laboratories, New York, NY) to the preparations prior to their application to the gradients. Following incubation, the samples were applied to the top of either 15-65% (HIX) or 15-55% (1869) sucrose gradients and centrifuged in SW-41 rotor at 36,000 rpsms at 10°C for 18 hours. The gradients were then fractionated into 0.5 ml aliquots
and assayed for reverse transcriptase activity or counted in a Beckman LS 333 scintillation counter to determine if radiolabeled RNA had been released from the virion core. The effect of cells on OIF/virus complexes were determined by the incubation of 5,000 cpm of either virus onto 50% confluent 35mm dishes containing FG-10 cells. Supernatants were collected following trypsin and heparin treatment and applied to sucrose gradients to determine radioactive profiles after centrifugation. Also, iodinated $^{125}$-labeled CHYLOs, LDLs, and HDLs were applied to similar sucrose gradients to localize the various lipoprotein fractions.

**Unlabeled MuLV chase and temperature studies in FG-10 cells**

The effect of whole mouse serum and purified serum lipoproteins diluted back to original serum volume were compared to anti-gp70 antibody in their ability to prevent the in vitro infection/transformation of FG-10 cells. Cells were preincubated at various times (5.0, 10.0, 15.0, 20.0, 30.0, 60.0, 90.0, and 120.0 minutes) with approximately 100 plaque-forming units (PFU) of either unlabeled 1869 or HIX MuLV. Virus was then washed three times with complete medium and followed by the addition of 1,000 inactivating units of either one of the previously purified lipoprotein fractions or anti-gp70 antibody diluted in complete medium (1.0 ml/dish). These fractions were allowed to incubate for 30 minutes prior to the addition of maintenance media. These studies were done at both 37°C and 4°C to investigate the effect of temperature on this interaction.
OIF, adsorption/penetration studies with $[^{35}S]$-labeled MuLV

Tissue culture dishes measuring 35 x 10mm (Falcon, Division of Becton, Dickinson and Co., Oxnard, CA) were plated with PG-10 cells at a concentration of 1,000,000 cells/dish and used after an overnight incubation. Radiolabeled HIX or 1869 virus (1,000 PFU or approximately 10,000 cpm) were added to equal volumes of either complete media or pre-determined concentrations of CHYLO/VLDLs (approximately 1.5 μg) and HDLs (approximately 30.0 μg) to effect greater than 90% inactivation of the OIF-sensitive HIX MuLV. This determination was very important so as to not saturate the effect of OIF with free virus. For comparison, these same viruses were incubated with neutralizing anti-gp70 antiserum diluted between 1/2,000 and 1/5,000, depending on the type of MuLV used to achieve greater than 90% neutralization. Finally, an artificial lipoprotein control consisting of protein phospholipid, cholesterol, and triglyceride (Intralipid, Cutter Laboratories, East Brunswick, NJ) was used at a concentration approximating those of CHYLOs (phospholipid - 20.0 mg/ml, cholesterol - 15.0 mg/ml, and triglyceride - 200 mg/ml). This was included to determine the effect of synthetic lipoprotein-like vesicles on viral adsorption. Following a 30 minutes incubation at 37°C, the media was removed from the dishes and 1.0 ml (total reaction volume) was added to duplicate plates, including those to be evaluated for biologic activity (OIF plaque-forming reductions). At indicated time intervals (Tn. 5, 15, 30, 60, 120, 180, and 240 minutes) the reaction mixture was removed and the dishes washed four times with 1.0 ml of phosphate-buffered saline (PBS). This material
was then pooled with the original reaction mixture and represented the unbound labeled virus. Following the washes, the dishes received 2.5 mg/ml (2.0 ml/dish) of trypsin (Difco, Detroit, MI) for 20 minutes on ice, after which the loosened cells were collected, centrifuged at 150 x g for 5 minutes at 10.0°C and washed once with PBS. This wash was then pooled with the trypsin supernate and represented that virus bound but not internalized. The cell pellet was then lysed with 1.0 N NaOH with the radioactivity in this fraction representing the internalized labeled virus.

**Assays of infectious virus - the OIF assay**

Assays for mouse serum or its purified OIF fractions have been previously described (9). Briefly, a dilution of mouse serum or its purified fraction was incubated with approximately 200 focus-forming units (FFUs) of either labeled or unlabeled virus for 1 hour at 25°C and the resulting mixture plated on the appropriate S+L- indicator cells (FG-10). Titers were expressed in 50% inactivating units. The relationship of LDIF concentration was linearly related to virus-surviving fraction \( \frac{V_n}{V_o} \) (9). Accordingly, OIF units O.U. = \((0.5) \left( \frac{1}{V_n/V_o} \right) / \text{dilution}\). The unit value could be plotted on a log-log paper using \( V_n/V_o \) values at several serum dilutions. Only values greater than 50.0 O.U./ml are considered specific due to the non-specific lipoprotein/lipid effect previously noted (Nara et al., unpublished observations).
RESULTS

Characterization and distribution of OIF from mouse serum

Serum lipoproteins from fasted animals were separated by differential ultracentrifugation into four major density fractions: densities less than or equal to 1.006 g/ml, between 1.006 and 1.063 g/ml, 1.0063 to 1.21 g/ml, and greater than 1.21 g/ml. Chemical composition of the isolated lipoproteins are presented in Table 2.1. CHYLO/VLDLs, \( d < 1.006 \), having total serum lipoprotein percentage composition of 4.5% and 13.0%, respectively, are characterized by their large amounts of triglyceride (76.0% by weight) and agarose gel electrophoretic mobilities described as: origin (CHYLOs), or pre-beta (\( \beta \)) (VLDLs) (data not shown). In fasted serum the lipoprotein class isolated with a density of \( \leq 1.006 \) g/ml will be termed CHYLO/VLDL. The intermediate density class lipoproteins (\( d = 1.006-1.063 \)), have a serum percentage composition of 4.8%, contain the intermediate and low density lipoproteins (IDL/LDL) and are characterized by a 3- to 4-fold increase of both cholesterol and protein (as compared to the \( d \leq 1.006 \) fraction), 3-fold decrease in triglyceride levels, and contain heterogenous particles with both alpha (\( \alpha \)) and beta (\( \beta \)) electrophoretic mobilities. The third class, \( d = 1.063-1.021 \) g/ml, represent the HDLs. These lipoproteins are characterized by a percentage serum composition of 77.6%, contain a single migrating band (data not shown), consists of larger amounts of protein (48.6% by weight), and moderate amounts of cholesterol (20.5% by weight). Also, SDS-PAGE analysis of these fractions revealed apolipoprotein...
(APO) profiles as presented in Fig. 2.1. Apolipoproteins recognized are: APO B, A-IV, E, A-I, Cs, and A-II in appropriate size and amounts for their respective lipoprotein classes. All the aforementioned physical-chemical properties match those recently reported for mouse lipoproteins by Camus et al. (15) and LeBoeuf et al. (16). The bottom fraction, $d>1.21$, contain a very small amount of very high density lipoproteins and all the remaining serum proteins (i.e., albumin, alpha, beta 1, beta 2 gamma globulins).

The biological activity profile of the purified serum lipoproteins are presented in Fig. 2.2. The majority of the activity was found in two distinct fractions, $d<1.006$ (CHYLO/VLDLs) and $d=1.063-1.21$ (HDLs). The $d<1.006$ and $d=1.063-1.21$ contained approximately equal amounts of inactivating activity and ranged from 2,000 to 3,000 O.U./ml. The small amount of activity seen at the bottom of the gradient are due to contaminating CHYLOs that failed to be collected from the surface of the gradient by the fractionator (these were seen to stain in the wells of agarose gel). CHYLO/VLDLs APO concentration from these fractions (adjusted to original serum volume) averaged 10.0 $\mu$g/$\mu$l. Typically, a final concentration of 0.1 $\mu$g/$\mu$l would inactivate 100 FFUs of virus. HDL APO concentration averaged 30 to 50 $\mu$g/$\mu$l and could inactivate 100 FFUs of virus at a final APO concentration of 3.0 $\mu$g/$\mu$l.

Density gradient binding studies with OIF and radiolabeled virus

The binding and virolysis studies for the $^{3}$H-radiolabeled RM- and E-MuLV are presented in Table 2.2 and Fig. 2.3. Various lipoprotein concentrations were titrated for both HIX and 1869 MuLVs
(Table 2.2). The ecotropic (1869) virus displayed a noticeable concentration-dependent density shift with increasing CHYLO-VLDL concentrations (5.0 μl-200.0 μl). Doubling the CHYLO-VLDL concentrations with HIX-MuLV caused a progressive decrease (from fraction 10 to fraction 8) in density, however, increasing amounts of CHYLO/VLDs failed to progressively alter its density. Fig. 2.3A demonstrates the effects of OIF and anti-gp70 antibodies at optimal concentrations (as titered previously) on the RM-MuLV. The RM-MuLV had its buoyant density shifted from the control value of 1.13 g/ml (fraction 10) to a new value of 1.11 g/ml (fraction 8) by the CHYLO/VLDLs and 1.12 g/ml (fraction 9) by the HDLs. These density shifts were independent of the lipoprotein concentration tested and intermediate in value when compared to the 1869 values for the same concentration and lipoprotein class. Protein concentrations less than 2.5 μg failed to cause any shift in either of the viruses. Low density lipoproteins failed to alter the density of either virus at all concentrations used (data not shown). Iodinated CHYLO/VLDLs and HDLs, when applied to the gradients, uniformly banded at the top (fractions 1 and 2). The ecotropic 1869 normally found to band at a specific gravity of 1.15 g/ml (fraction 10), was shifted seven fractions to tube no. 3 (specific gravity of 1.08-1.10 g/ml), by the addition of 200 μl of CHYLO/VLDLs (Fig. 2.3B). No other density alterations were noted with any other lipoprotein fractions. The addition of anti-gp70 antibody which, served as one positive control, caused both viruses to increase their density by similar amounts, and indicated the capability of the assay system to determine binding
(Figs. 2.3A, 2.3B). The other control included detergent and complement-mediated lysis of the labeled virions, and resulted in the labeled ribonucleic acids to band at the top of the gradient (Figs. 3A, 3B). Reverse transcriptase activity in all cases were associated with labeled MuLVs and in only artifically lysed or complement-mediated virolysis was it found at the top of the gradients.

The effect of mild pronase pretreatment of both viruses prior to the addition of CHYLO/VLDLs are presented in Table 2.3 and were found to affect the OIF-induced buoyant density shifts. When used at concentrations found to affect the binding of anti-gp70 (data not shown), no density shifts occurred following the addition of CHYLO/VLDLs (Table 2.3) and HDLs (data not shown). Also, when OIF was pretreated with pronase and then incubated with virus, no difference in binding was detected (data not shown). The effect of anti-gp70 antibody either before or after the addition of CHYLO/VLDLs is presented in Table 2.4. Preincubation of either virus with anti-gp70, followed by the addition of increasing CHYLO/VLDLs concentration, failed to significantly alter the density of both classes of viruses when compared to the virus/antibody controls. The reverse of this experiment (also presented in Table 4) showed that the preincubation of virus with CHYLO/VLDLs, followed by the addition of increasing amounts of antibody, caused progressive increases in the density of both viruses (1.140 g/ml to 1.180 g/ml for HIX, and 1.152 g/ml to 1.190 g/ml for 1869). It thus appears that OIF is not blocking access to the gp70 molecule.
Finally, the effect of isolated CHYLO/VLDL from various species were investigated as to their ability to alter virus density (Table 2.5). The RM-MuLV density was shifted by the addition of various species of CHYLO/VLDL fractions. These caused similar density shifts for all the species tested, and ranged from 1.028 g/ml (rat) to 1.042 g/ml (rabbit). These alterations in buoyant density were seen with the RM-MuLV comparable to the nonspecific density shifts seen with E-MuLV when mouse CHYLO/ VLDLs were used. These same species CHYLO/VLDLs were also found to alter the density of the E-MuLV as well (Table 2.5). This finding supports the conclusion that dose-dependent buoyant density shifts are non-specific and seem to correlate with the absence of biologic activity.

The effect of various lipoprotein fractions in chase experiments at 37°C and 4°C (unlabeled virions)

The previous experiments suggested that binding was occurring without virolysis and, as such, suggest either a noncellular or cellular inactivation mechanism. Thus, it was of interest to study the kinetic relationships that existed between the various lipoproteins, cells and the virion. Preincubation of FG-10 cells with either CHYLOs or HDLs followed by three washes in complete medium failed to affect RM-MuLV infectivity of the cells (data not shown). However, the preincubation of virus with cells did serve to help characterize this interaction. In Fig. 2.4, the normal (37°C) entry kinetics are presented for both viruses. The RM-MuLV (OIF-sensitive) has a significantly faster wash resistant adsorption rate (Tn, 1/2 of 10 minutes) when compared to the E-MuLV rate (Tn, 1/2 of 120
minutes). Fig. 2.5 shows the results of various preincubations (Tn, 5.0, 10.0, 20.0, 30.0, 60.0, 90.0, and 120.0 minutes) of cells with virus, followed by the addition of one of the following: CHYLOs, HDLs, whole mouse serum, lipoprotein-depleted mouse serum, and anti-gp70 antisera. OIF lipoprotein fractions can affect a significant change in the final infectivity pattern as late as 60.0 minutes after virus pre-adsorption. The best lipoprotein fractions for inactivation following virus pre-adsorption are the CHYLOs and the HDLs. These fractions inactivated greater than or equal to (≥) 94% of RM-MuLV (HIX), following a preincubation of 0 to 30 minutes. Maximum inactivation (99%) occurred at a preincubation of 10 minutes and was still capable of 80% inactivation as late as 120 minutes.

When compared to comparably titered anti-gp70 antiserum, >93% of HIX was inactivated between 0 and 30 minutes. However, by 60 and 120 minutes, only 40% and 30% inactivations, respectively, were noted.

The effect of temperature on this cellular aspect of inactivation are presented in Fig. 2.6. The active lipoprotein fractions (CHYLO/VLDLs and HDLs), as well as the anti-gp70 antiserum (data not shown), were found to prevent infection of the cells equally as well following a preincubation of 120.0 minutes at 4°C. Times longer than this could not be evaluated due to temperature-related cytotoxicity.

The effect of various lipoproteins on the adsorption and penetration of $^{35}$S-labeled MuLVs

The effect of purified lipoprotein fractions on the adsorption and penetration of radiolabeled MuLVs are presented in Fig. 2.7, 2.8
and 2.9. A maximal level of total cellular binding (approximately 47%) occurred by 4 hours for RM-MuLV (Fig. 2.7), with 83% of the virions binding by 1-2 hours. The absolute amount of virus bound was proportional to the amount added and varied between 35 and 55%, depending on the virus preparations. Thus, it appears that binding followed reasonably well the lines of an exponential function with a half-time of approximately 15 to 30 minutes and paralleled the kinetic pattern seen in Fig. 2.4. The incubation of CHYLOs and HDLs with the RM-MuLV appear to enhance slightly (approximately 2-10%) the cellular binding between 5.0-120.0 minutes, after which the total binding could not be distinguished from that of virus controls. Anti-gp70 serum prevented as much as 89% of the RM-MuLV from binding to the cells. Radiolabeled E-MuLV behaved in similar fashion to its unlabeled counterpart (Fig. 2.4) by exhibiting slower cell binding kinetics (half-time of 120.0 minutes). Slightly less (approximately 30%) E-MuLV was found to bind by 4 hours when compared to the RM-MuLV (maximal binding occurred by 6 hours (data not shown). A slight increase in total binding also occurred with this virus following preincubation with CHYLOs and HDLs (Fig. 2.7). The anti-gp70 antiserum had dissimilar pre-adsorbing effects (approximately 80-85% of virus was prevented from binding). The synthetic lipid had no significant effect on binding of either virus at any time-point tested (data not shown).

The internalization profiles are presented in Fig. 2.8. A 10- to 13-fold decrease in internalization (trypsin resistance) for the RM-MuLV was observed for CHYLOs and HDLs, respectively, by 5.0
minutes. By 30.0 minutes, however, the amount of internalized CHYLO-treated virus becomes indistinguishable from cells receiving just virus and is only slightly lower (1-2% per time-point) throughout the remaining experimental period. The HDLs, however, were found to significantly alter the internalization process throughout the experimental period and ranged between a maximal value at 5.0 minutes (13-fold decrease) to a 4-fold decrease (60.0 minutes), to a final value at 240.0 minutes (6-fold decrease). Due to the small amount of virus that bound in the face of anti-gp70 antisera, conclusions about the amount internalized cannot be made. The E-MuLV (Fig. 2.8) internalization profile was unaltered by any of the lipoprotein fractions tested when compared to controls and characterized by paralleling lines at all time-points.

The specific biological activity data are presented in Fig. 2.9. Presented data indicate that the radiolabeled MuLV was infectious as characterized by the presence of 200 to 250 foci per dish in those plates receiving only $^{35}$S-labeled RM- and E-MuLV, respectively. Also demonstrated was the similarity of unlabeled virus cellular entry kinetics (Fig. 2.4) with the total cellular binding of radiolabeled MuLVs (Fig. 2.7). Finally, Fig. 2.10 shows the similar inactivating effect of both CHYLOs and HDLs specifically on the RM-MuLV at all incubation times tested. Also, it can be concluded that the internalized CHYLO-treated RM-MuLV noted at 30.0+ minutes consisted of inactivated virus. The E-MuLV was uniformly resistant to the factors. Thus, it appears that the radiolabeled virus modeled the unlabeled MuLVs with respect to adsorption/penetration kinetics and
OIF susceptibility, and allow for interpretations to be made concerning the effects of OIF on the various stages of viral adsorption and penetration.
DISCUSSION

A variable serum distribution for OIF has been suggested by many authors (8,9) and probably reflects either the dietary status of the animal or the method of isolation/purification used. In this study, control of diet and a sensitive method for lipoprotein isolation were used to generate very reproducible profiles of serum lipoproteins having OIF activity. The distribution of this serum factor resides or co-purifies in the CHYLOs and HDLs and compares, in both the recently published physical/chemical properties (15,16) and biological activity profiles previously suggested by various authors for these lipoprotein classes (8,17).

Although much effort has been invested to characterize the factor, no studies have been published to elucidate the mechanism of virus inactivation. The factor has been suggested by Kane et al. (1979) to be an apolipoprotein (i.e., C), a protein constituent of CHYLOs and HDLs capable of transferring to ordered artificial lipoproteins or human CHYLOs. The actual physical/chemical evidence for this was lacking, and was inferred by the author from transfer studies which traced the biologic activity of the material. It is well known that apolipoprotein C is present in these lipoprotein fractions and transfer of apolipoproteins between various lipoprotein classes in other well-characterized systems has been previously demonstrated (18). Thus, identity of the factor still awaits satisfactory characterization.

The mechanism of virus inactivation by OIF appears to be viro-static in nature. The radiolabeled binding studies clearly indicate
that labeled viral RNA and viral reverse transcriptase are not released following incubation with active fractions alone or with these complexes or in the presence of cells. Although the binding appears to be non-specific (both classes of viruses bind CHYLOs) and possibly mediated by the gp70 envelope protein, the mouse CHYLO fractions, when incubated with RM-MuLV, were shifted to a lesser intermediate density value on the gradient when compared to the larger decrease in density that occurred with the E-MuLV. Following the addition of CHYLOs from other species (also known not to inactivate RM-MuLV), the RM-MuLV was shifted in much the same fashion as the E-MuLV. Thus, suggesting these large density alterations to be non-specific and reflect the natural spontaneous lipid/lipoprotein interactions which do not involve virus inactivation. Also, the HDL fraction from mouse serum shifted only the RM-MuLV. In addition, the RM-MuLV binding titration study indicates that, over a wide range of CHYLO concentrations, the density shift remains constant, whereby the E-MuLV density varied proportionally to the amount of CHYLOs added from any of the species tested. It should be mentioned that the CHYLO/VLDLs generated by these methods are a very uniform population of lipoproteins based on similar size and density (15,18). It therefore seems that, if this binding were non-specific, the density shift noted for the RM-MuLV would be similar to that recorded for the E-MuLV. Either fewer lipoprotein particles are binding to the RM-MuLVs or alteration in buoyant density is occurring following the interaction with virus. The electron micrographs included (Fig. 2.11), are from samples generated during a pilot study, done in
similar fashion (as the binding studies), and suggest the latter hypothesis. The interesting observation noted was that both viruses bind the CHYLOs (as suggested by the binding studies), but that the CHYLO particle size and condition of the virus envelope are different. The average size of the CHYLO/VLDLs attached to the E-MuLV are approximately 100 nm in diameter with no apparent alteration in the viral envelope. The CHYLO/VLDLs attached to the RM-MuLV, however, are uniformly smaller, approximately 40 nm (similar in size to HDLs), and appear to be blending or melting the viral envelope. These profiles were selected because they were both taken at the same time period and found to be attached to the cell surface. Only a limited number of CHYLO/virus/cell complexes were found in this study and thus, definitive morphologic studies will be required. Regardless, this morphologic alteration may serve as a clue to explain the difference in density noted in the binding studies and suggests a morphologic alteration associated with biologic activity. More specifically, buoyant density of the lipoproteins are directly related to their overall mean diameter (correlating with the amount of triglyceride) and thus, given that the population of CHYLOs used were of uniform diameter (examined under the electron microscope), a noticeable size-related morphologic alteration occurred and may explain the difference in density shifts associated with the RM-MuLV. This may remotely suggest a viral envelope-associated enzyme undergoing activation by the appropriate substrate (receptor) associated with the CHYLO particle. This may further suggest that retroviral envelopes containing host cell membrane protein receptors/enzymes are
recognized by, and bind to, circulating lipoproteins. Similar ultrastructural morphologic (envelope melting) changes have been seen in ongoing studies involving the adsorption/penetration phase of this virus in S+L- mouse fibroblasts. It is not known yet if this interaction occurs in the absence of the cell membrane, however, studies are currently investigating this possibility. Many attempts were undertaken to separate the OIF-virus complexes but, due to the exquisite temperature lability of the RM-MuLV, suggesting an envelope enzyme, biologic activity of the virus could not be evaluated.

Though the biologic activity of the OIF RM-MuLV complexes are not known, it is now known from our chase and temperature studies that inactivation takes place on the cell membrane. Different rates of cellular entry have been suggested for the various classes of murine retroviruses (19-21). So is the case observed for our E- and RM-MuLV. The RM-MuLV was found to have significantly faster rate of adsorption based on number of foci generated per unit-time following multiple post-adsorption washes. Though the adsorption rate is rapid (Tn, 1/2 of 10 minutes), 95% or more of the virus could be inactivated by OIF and anti-gp70 following a 15 minutes virus preincubation period. Following a 60 minutes virus preincubation period, however, OIF fractions were still capable of inactivating 90% of the virus as compared to only 40% inactivation by hyperimmune anti-Friend MuLV antibody. The internalization data suggest that 60% of the bound virus is trypsin-resistant by 15 minutes, yet the OIF lipoprotein fractions are able to affect virus inactivation. This suggests that OIF can affect virus somewhere between adsorption and cytoplasmic
internalization (traditionally defined by trypsin resistance), or CHYLOs and HDLs can inactivate RM-MuLV after the internalization stage. Handelin et al. (21) found HIX-sensitized cells to be uniformly resistant to cytotoxic antisera after 45 minutes incubations and concluded that rapid internalization of the virus and/or low receptor concentrations or avidities to be the cause. This observation suggests that much of the envelope gp70 is already beyond the stage of simple adsorption and argues that the RM-MuLV is being activated by OIF at a stage beyond which traditional antibody may affect. CHYLOs are known to interact traditionally at the cell membrane through their apolipoprotein receptor(s) with activation of a membrane-bound lipoprotein lipase which promotes lipolysis (18). There is more evidence that VLDLs, and especially HDLs, can undergo receptor-mediated endocytosis and thus involve the cytoplasmic compartment more directly.

The temperature study clearly suggests that RM-MuLV adsorption is unaffected by 4°C, but viral inactivation by OIF can be prolonged much beyond the normal 37°C OIF-resistant window. OIF, when compared to antibody, have very similar effects on early stage post-adsorption virions, however, a number of significant differences exist. First, OIF causes a decrease in the virion buoyant density and antibody increases it; second, the addition of complement to OIF does not enhance virolysis; third, OIF appears not to be able to alter the virus buoyant density following pretreatment with antibody, however, antibody does appear to alter the virus density following pretreatment with OIF; fourth, pronase treatment of virus prior to the
addition of OIF, eliminates the binding of both OIF and anti-gp70 and again suggests the importance of envelope proteins; fifth, and possibly most important, antibody prevents viral adsorption, whereas OIF tends to enhancing it; and last, antibody does not chase (inactive) pre-adsorbed virions as well or as long as OIF.

It appears quite obvious that the effect of OIF on just the virus particle itself is academic. This is based on the fact that, (1) no virolysis occurs, (2) RM-MuLV/OIF complexes bind to cells with as much or more affinity than just the virus itself, and (3) inactivation can occur so late in the penetration stage. This inactivating potential gives OIF an attractive therapeutic potential should isolation of the factor prove efficacious for other retroviral-related diseases.

Finally, it is interesting to note that only HDLs were responsible for significantly inhibiting the trypsin-resistant phase of penetration (internalization). HDLs are known to have high affinity cellular receptors for their uptake (18) and one could speculate on the possible association of these receptors with those required by, or associated with, this retrovirus for adsorption/penetration into the host cell.
SUMMARY

Lipoprotein-associated oncornavirus inactivating factor (OIF) present in mouse serum is associated with the following lipoprotein classes: chylomicrons, very low density lipoproteins (CHYLO/VLDLs) and high density lipoproteins (HDLs). CHYLO/VLDLs were found to bind to both ecotropic (E-) and recombinant murine leukemia viruses (RM-MuLV). However, only CHYLO/VLDLs and HDLs were found to bind in a unique density and lipoprotein class-specific manner to the RM-MuLV. This binding did not cause the release of labeled ribonucleic acids or reverse transcriptase from the virion core either alone or in association with cells. Cellular binding of $^{35}$S-labeled E- and RM-MuLV-OIF complexes were found to bind with equal or greater affinity to cells compared to labeled virus controls. High titered anti-gp70 antiserum, however, prevented E- and RM-MuLV from adsorbing to the cells. In chase studies at 37°C, both CHYLO/VLDLs and HDLs could inhibit foci production by 90% in RM-MuLVs given up to a 60 minutes preincubation. When compared again to anti-gp70 goat serum, only 50% foci reductions were observed. $^{35}$S-labeled MuLV studies indicated greater than 60% of the bound virus is trypsin resistant (internalized) by this time period. Thus indicating OIF's ability to inactivate the infectious virion late in the penetration phase. Also, RM-MuLV adsorbed to the cells and held at 4°C for 120 minutes could later be inactivated by both OIF and anti-gp70 antisera. Finally, HDLs were shown to significantly reduce the internalization rate for RM-MuLV throughout the experimental period (4 hours).
CHYLO/VLDLs were also effective at preventing early internalization (80% up to 15 minutes), however, internalization rates at later time-points approached those of the control virus. It appears that OIF inactivation of murine leukemia retroviruses represents a unique antiviral host mechanism capable of affecting the virus late in the penetration phase.
Table 2.1 Chemical Composition of Mouse Lipoproteins

<table>
<thead>
<tr>
<th>Component</th>
<th>d&gt;1.006 (CHYLO/VLDL)</th>
<th>d=1.006-1.063 (IDL-LDL)</th>
<th>d=1.063-1.021 (HDL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>7.8</td>
<td>21.4</td>
<td>48.5</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>10.2</td>
<td>26.1</td>
<td>30.0</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>76.0</td>
<td>28.5</td>
<td>1.5</td>
</tr>
<tr>
<td>*Cholesterol ester</td>
<td>2.9</td>
<td>19.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>3.1</td>
<td>6.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>6.0</td>
<td>25.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

*Values are expressed as the percent composition by weight and coefficients of variation for the chemical measurements were within 5% for repeat isolations.

*a,bCholesterol ester values obtained by subtracting free cholesterol from total cholesterol.
Table 2.2 CHYLO/VLDL Binding Titration Study with Ecotropic and Recombinant MuLVs.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>HIX</th>
<th>1869</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus only</td>
<td>1.130&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.56</td>
</tr>
<tr>
<td>Virus only + 5.0 µl CHYLO/VLDL</td>
<td>1.113</td>
<td>1.142</td>
</tr>
<tr>
<td>Virus only + 25.0 µl CHYLO/VLDL</td>
<td>1.112</td>
<td>1.138</td>
</tr>
<tr>
<td>Virus only + 50.0 µl CHYLO/VLDL</td>
<td>1.112</td>
<td>1.13</td>
</tr>
<tr>
<td>Virus only + 100.0 µl CHYLO/VLDL</td>
<td>1.110</td>
<td>1.09</td>
</tr>
<tr>
<td>Virus only + 200.0 µl CHYLO/VLDL</td>
<td>1.119</td>
<td>1.07</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number indicates the specific gravity of the fraction collected from the sucrose gradients, the units are in grams/ml. The density of the gradient was linear, with Δd=0.012 g/ml/0.5 ml fraction for HIX gradient and Δd=0.014 g/ml/0.5 ml fraction for the 1869 gradient.
Table 2.3 Effect of Pronase on OIF/MuLV Binding

<table>
<thead>
<tr>
<th>Protocol</th>
<th>HIX</th>
<th>1869</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus only</td>
<td>1.132(^a)</td>
<td>1.154</td>
</tr>
<tr>
<td>Virus + pronase</td>
<td>1.132</td>
<td>1.154</td>
</tr>
<tr>
<td>(Virus + pronase) + CHYLO/VLDL</td>
<td>1.142</td>
<td>1.159</td>
</tr>
<tr>
<td>(Virus + CHYLO/VLDL) + pronase</td>
<td>1.083</td>
<td>1.119</td>
</tr>
<tr>
<td>Virus + CHYLO/VLDL only</td>
<td>1.112</td>
<td>1.109</td>
</tr>
</tbody>
</table>

\(^a\)Number indicates the specific gravity of the fraction collected from the sucrose gradients, the units are in grams/ml. The density of the gradient was linear, with \(\Delta d=0.012 \text{ g/ml}/0.5 \text{ ml} \) fraction for the HIX gradient and \(\Delta d=0.014 \text{ g/ml}/0.5 \text{ ml} \) fraction for the 1869 gradient.
Table 2.4 Effect of Anti-gp70 Antisera on OIF/MuLV Binding

<table>
<thead>
<tr>
<th>Protocol</th>
<th>HIX</th>
<th>1869</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus only</td>
<td>1.132&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.158</td>
</tr>
<tr>
<td>Virus + CHYLO/VLDL</td>
<td>1.119</td>
<td>1.109</td>
</tr>
<tr>
<td>(Virus + OIF) + 1x Ab</td>
<td>1.142</td>
<td>1.148</td>
</tr>
<tr>
<td>(Virus + OIF) + 2x Ab</td>
<td>1.146</td>
<td>1.153</td>
</tr>
<tr>
<td>Virus + OIF) + 10x Ab</td>
<td>1.164</td>
<td>1.172</td>
</tr>
<tr>
<td>(Virus only) + 10x Ab</td>
<td>1.181</td>
<td>1.192</td>
</tr>
<tr>
<td>(Virus + Ab 1x) only</td>
<td>1.167</td>
<td>1.188</td>
</tr>
<tr>
<td>(Virus + Ab 1x) + 1x CHYLO/VLDL</td>
<td>1.168</td>
<td>1.189</td>
</tr>
<tr>
<td>(Virus + Ab 1x) + 10x CHYLO/VLDL</td>
<td>1.169</td>
<td>1.186</td>
</tr>
<tr>
<td>(Virus + Ab 1x) + 20x CHYLO/VLDL</td>
<td>1.170</td>
<td>1.190</td>
</tr>
<tr>
<td>Virus + 20x CHYLO/VLDL only</td>
<td>1.102</td>
<td>1.107</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number indicates the specific gravity of the fraction collected from the sucrose gradients, the units are in grams/ml. The density of the gradient was linear, with \( \Delta d = 0.012 \text{ g/ml/0.5 ml fraction for the HIX gradient and } \Delta d = 0.014 \text{ g/ml/0.5 ml fraction for the 1869 gradient.} \)
Table 2.5 Effect of Various Species CHYLO/VLDL on MuLV Binding

<table>
<thead>
<tr>
<th>Protocol</th>
<th>HIX</th>
<th>1869</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus only</td>
<td>1.131&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.151</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.042</td>
<td>1.099</td>
</tr>
<tr>
<td>Cat</td>
<td>1.032</td>
<td>1.030</td>
</tr>
<tr>
<td>Rat</td>
<td>1.028</td>
<td>1.026</td>
</tr>
<tr>
<td>Goat</td>
<td>1.031</td>
<td>1.025</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number indicates the specific gravity of the fraction collected from the sucrose gradients, the units are in grams/ml. The density of the gradient was linear, with $\Delta d=0.012$ g/ml/0.5 ml fraction for the HIX gradient and $\Delta d=0.014$ g/ml/0.5 ml fraction for the 1869 gradient.
Fig. 2.1 A 13% sodium dodecyl sulfate-polyacrylamide gel elec­
trophoresis analysis of potassium bromide purified mouse serum
lipoproteins stained with Coomassie Brilliant Blue G-250.
Location of the major apolipoprotein bands are indicated on
the right hand edge. Lane 1, molecular weight standards;
Lane 2, chylomicrons (d ≤1,006 g/ml); Lane 3, very low
density lipoproteins (d <1.006 g/ml); Lane 4, low density
lipoproteins (d=1.006-1.063 g/ml); Lane 5, high density
lipoproteins (d=1.063-1.021 g/ml). Lane 2 represents the
predominant lipoprotein species present during oil-induced
lipemia. Lane 3 represents the predominant lipoprotein
species during an 18 hour fast.
Fig. 2.2 Inactivation profile of potassium density gradient purified lipoprotein factions from fasted whole mouse serum. CHYLO/VLDL fraction is found in the $d=1.006$ g/ml region, the low density lipoproteins are found in the $d=1.006-1.063$ g/ml region, the high density lipoproteins are found in the $d=1.063-1.21$ g/ml region, and the very high density lipoproteins and other serum proteins are found in the remaining fraction.
Neutralization Profile of KBr Density Gradient
Whole BALB/c Sera (18 hr fast)

Fig. 2.2
Fig. 2.3 Buoyant density shift assay to determine binding characteristics of OIF to $^3$H-labeled MuLV virions. The effect of OIF on RM-MuLV (15-55% sucrose gradient) and E-MuLV (15-65% sucrose gradient) buoyant density are presented in panel A and B, respectively. The symbols are similar for both viruses and are: virus only (0); virus + CHYLO/VLDL (□); virus + HDLs (Δ); virus + anti-gp70 antibody (◊); and virus + anti-gp70 antibody and complement (0—0).
Fig. 2.3
Fig. 2.4 Comparison of RM-MuLV and E-MuLV entry kinetics into FG-10 cells. RM-MuLV (▲) or E-MuLV (●) are pre-adsorbed for various time-points, extensively washed, and maintenance media added. The resultant foci are counted and represented as the % of the total foci formed.
Fig. 2A

- ○ 1869 Only
- □ 1869 + Chylo/VLDL (100 λ)
- △ 1869 + HDL (100 λ)
- ◇ 1869 + Anti-GP70 Antibody
- ○ 1869 + Anti-GP70 Ab + C'

Fraction No.

cpm

1,000
800
600
400
200
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Fig. 2.4
Fig. 2.5 Kinetics of OIF compared to anti-gp70 antibody in RM- and E-MuLV chase studies. Virus was preincubated at 37°C on indicator cells (FG-10) for various time periods, washed extensively, followed by the addition of OIF or antibody (60 minutes incubation), and finally, maintenance media added. Six days later the plates were counted and foci enumerated and represented by the virus surviving fraction \( \frac{V_n}{V_0} \) under varying pre-adsorption times. The various reactants are represented as: CHYLO/VLDL + RM-MuLV (□); HDL + RM-MuLV (■); RM-MuLV + anti-gp70 (○); E-MuLV + anti-gp70 (●); whole mouse serum + RM-MuLV (△); and lipoprotein-deficient mouse serum + RM-MuLV (▲). Lipoprotein-deficient mouse serum was derived from the d >1.21 g/ml fraction of the potassium bromide gradient.
Fig. 2.6 Kinetics of OIF in RM-MuLV 4°C chase studies. FG-10 cells were cooled to 4°C and virus was preincubated, extensively washed, 37°C OIF added (incubated at 37°C for 60 minutes), and finally, maintenance media added. Six days later the foci were counted and plotted here as the number of foci per dish. The reactants are represented as: RM-MuLV only (△); RM-MuLV + CHYLO/VLDL (◇); and RM-MuLV + HDL (●).
4°C Binding Chase Study With Purified Lipoprotein Fx’s (RM-MuLV)

Fig. 2.6
Fig. 2.7 Binding of $^{35}$S-labeled MuLV to FG-10 cells in the presence of OIF or anti-gp70 antibody. The effects of OIF and/or antibody on RM- and E-MuLVs are presented in panel A and B, respectively, and represented by: virus only (O); virus – CHYLO/VLDL (▲); virus + HDL (O); and virus + anti-gp70 (■). Virus (10,000 cpm) + OIF or antibody were mixed, incubated and plated for various time intervals (see Materials and Methods for details). The abscissa represents the percentage of trypsin-sensitive $^{35}$S-MuLV bound to cells as compared to the total amount added.
Fig. 2.7 Hours % E-MuLV Bound to Cells (total)

% E-MuLV Bound to Cells (total)

GF-MuLV Only

HL6s + Virus

CMV Virus

E-MuLV Only

Fig. 2.7 Hours % RM-MuLV Bound to Cells (total)

% RM-MuLV Bound to Cells (total)

GF-MuLV Only

HL6s + Virus

CMV Virus

RM-MuLV Only

SS-1689/LDIF Binding Study

SS-1689/LDIF Binding Study
Fig. 2.8 Internalization of $^{35}$S-labeled MuLV to FG-10 cells in the presence of OIF and anti-gp70 antibody. The effects of OIF or antibody on RM- and E-MuLV are presented in panel A and B, respectively, and represented by: virus only (○); virus + CHYLO/VLDL (▲); virus + HDL (○); and virus + anti-gp70 (■). The abscissa represents the percentage of trypsin-resistant (internalized) $^{35}$S-MuLV as compared to the total amount bound for each time-point.
Fig. 2.9 Biologic activity of $^{35}$S-labeled MuLV used in cellular binding study. Duplicate plates as used in Fig. 2.7 were rinsed, not trypsin treated, given maintenance medium and foci counted on day 6. The abscissa represents the percentage of foci in the virus controls as compared to the various reactants and are represented by: RM-MuLV only (○—○); E-MuLV (●—●); RM-MuLV + CHYLO/VLDL (■—■); RM-MuLV + HDL (▲—▲); E-MuLV + CHYLO/VLDL (■—■); and E-MuLV + HDL (△—△).
$^{35}$S-Labeled MuLV/LDIF, Effect on Foci Formation

- RM-MuLV Only
- E-MuLV Only
- Chylo + Rm-MuLV
- HDL + Rm-MuLV
- Chylo + E-MuLV
- HDL + E-MuLV

Fig. 2.9

% of Total Foci/Dish

Time (hours)

15' 30' 1 2 3 4
Fig. 2.10 Transmission electron micrograph (magnified 90,000X) showing the various ultrastructural effects of CHYLO/VLDLs on MuLVs. (A) represents the E-MuLV attached to the cell membrane (following a 5 minute incubation) and rosetted by uniformly shaped and sized lipoproteins. (B) represents the RM-MuLV (OIF-sensitive) at the same time-point, also attached to the cell membrane but rosetted by variably sized and shaped lipoproteins.
REFERENCES


15. Camus, M., Chapman, M.J., Forgez, P., and Laplaud, P.M. Distribution and characterization of the serum lipoproteins and


CHAPTER III

ACTIVATION OF MURINE RETROVIRUS-INACTIVATING FACTOR
IS DEPENDENT ON THE VASCULAR COMPARTMENT

In view of the recent discovery of a family of human retrovirus, their causal relationship to disease (1,2), and their obvious parallels to retroviruses of other species (1-3), investigations involving host retroviral controlling mechanisms may provide a key understanding about their biology and possibly yield other nonconventional therapeutic modalities. It is well known in the mouse species that a lipoprotein associated serum factor (OIF), transferable to human high density lipoproteins (4), is present at high titers (depending on dietary status), and will inactivate all xenotropic (X-), recombinant (RM-) and some polytropic murine leukemia viruses (MuLVs) (5,6) (Table 3.1). It has also been suggested that OIF plays a significant role in host retroviral controlling mechanisms, as evidenced by the uniform absence of in vivo isolatable X-MuLVs and the appearance of genonomically masked MuLV variants (OIF resistant (7,8). An analogous situation may currently exist in the human. Increasing evidence suggests the presence of equivalent endogenous retroviral sequences of murine (9-11) and nonhuman primate origin in human cells (12,13), and their potential association with disease,
i.e., autoimmune connective tissue disorders (14-16). They may serve as oncogenic targets of recombination for the previously mentioned exogenous HTLV family. Therefore, should OIF be intimately related to control or expression of endogenous retroviruses it can be expected to be an applicable thesis to many if not all species.

Recently OIF has been shown to be associated with three major classes of serum lipoproteins, i.e., Chylomicrons (CHYLOs), very low density lipoproteins (VLDLs) and high density lipoproteins (HDLs) (6; Nara et al., unpublished observations). CHYLOs which are the biologically most active, are synthesized in the enteron while the other two classes are generally considered to be synthesized in the liver (18). Most dietary triglycerides are transported in the plasma as CHYLOs. Triglycerides are hydrolyzed in the duodenum to free fatty acids and 2-monoglycerides; after absorption by the enterocyte, triglycerides are then resynthesized by the smooth endoplasmic reticulum and incorporated into a lipoprotein bilayer micelle which are then released at the lateral cell surface into the lymphatic lacteal. The CHYLOs then travel up the intestinal lymphatics to the main thoracic lymph duct and mix with the blood in the region on the left external jugular and cranial vena cava. Due to the enhanced biologic activity of serum CHYLOs and the obvious variation in anatomic site-related synthesis, we decided by segmental microannulation studies to investigate the origin of OIF in the mouse.

To do this, adult BALB/c mice were fasted for 18 hours, given 1/2 cc of Lipomul (The Upjohn Co., Kalamazoo, MI), and allowed 60 minutes for digestion. The terminal surgical procedure used was as
described by Boak and Woodruff (19); however, an inhalant anesthetic (Metafane) was used "to effect" via a nose cone. At this time, the intestinal lymphatics were clearly seen as white anastomosing vessels. The two sites selected for cannulation, were the main intestinal lymphatic trunk and the main thoracic lymphatic duct (Fig. 3.1). The former was selected due to size constraints and to determine if the draining mesenteric lymph node had an effect on the production of OIF. The thoracic duct was included in the studies after the negative results obtained by intestinal lymphatic studies.

Approximately 300 μl of intestinal lymph (IL) was collected over a 2-hour interval. The thoracic duct, however, provided 600 μl per hour of lymph (TDL) and could be collected in terminal cannulations for up to 6 hours. Along with these lymph samples, simultaneous blood samples were collected from the orbital sinus and were tested for comparative OIF activity. Also tested were surviving, chronically cannulated normal F₁ C57BLG/CBA-CA mice. These animals provided large volumes of thoracic duct lymph (TDL) and were graciously donated by Dr. Jonathan Sprent along with parallel serum samples.

The lymph was tested initially as whole undiluted lymph from the intestinal and thoracic duct sites and are presented in Table 3.2. No OIF activity (<50 O.U./ml) was detected in either site; however, minimal activity (50-100 O.U./ml) was associated with whole thoracic duct lymph (TDL) and was later determined to be associated with contaminating (recirculating) serum HDLs, known to have OIF activity. The serum, however, from these same animals were found to contain ample amounts of OIF, which increased proportionally with the degree
of chylomicronemia present in the serum (Fig. 3.2). To examine the possibility of an anti-OIF factor present in lymph, cells were removed by centrifugation at 100 x g for 5 minutes and then purified by three potassium bromide gradient (KBr) ultracentrifugations (19). Thoracic duct CHYLOs (TDC) were then rediluted back to original lymph volume, dialyzed extensively against phosphate buffered saline and retested for OIF activity. As can be seen in Fig. 3.3, although sequential centrifugation removes large amounts of protein, it has no "activating" OIF effect. When serum derived chylomicrons are sequentially purified by 3 KBr gradients (Fig. 3.3, lanes 4 and 4), they also experience this protein shedding; however, they retain most of their biologic OIF activity.

TDL, purified TDC, IL and serum CHYLOs were tested for their comparative chemical compositions (Table 3.2). Undiluted TDL was found to contain 4-500 times the concentrations of the various major lipoprotein constituents of serum. Also, when intestinal lymph was diluted to equivalent concentrations based on turbidity, their chemical compositions were similar but tremendous differences were noted in their OIF activity.

To determine the simple effects of TDC mixing with just the blood components, TDC were diluted in equal volumes with heat-inactivated and nonheat-inactivated fasted whole serum. The serum was fasted to reduce OIF levels associated with serum CHYLOs. These mixtures were then incubated at 37°C for 90 minutes so as to mock the in vivo situation of oil gavage followed by terminal bleeding for acquisition of high titered OIF sera. When tested, a simple
dilutional profile for the serum was seen in both the fasted, heat- and nonheat-inactivated whole serum (Table 3.3). More specifically, if OIF activation of TDC requires only the blood cellular/serum components, then TDC added in equal volume to the fasted serum should enhance the OIF titers comparable to an equivalent amount of serum CHYLOs. However, when chemically equal amounts of TDC and serum CHYLOs were added to fasted serum, no enhancement of the TDC/fasted serum occurred, suggesting that the tremendous activation of serum CHYLO OIF values seen post-gavage is not simply associated with the serum compartment. Also, heparinized whole blood was mixed with TDC and also found to not cause OIF activation. These findings strongly suggest that activation may require the endothelium with or without the association of blood cellular/serum components.

Apolipoprotein profiles from 3 KBr purified TDC and serum CHYLOs were compared and are presented in Fig. 3.3. TDC were found to contain increased amounts of apolipoprotein A-IV (46,000 daltons) and A-I (28,000 daltons) and a faint band at 10 to 12,000 daltons. This apolipoprotein profile has been reported previously in other species (20-22) and is characteristic of newly formed and secreted CHYLOs. These SDS-PAGE gels were evaluated for protein content after Coomassie blue and silver stains to insure that all protein species were being detected. Also, purified TDC and serum CHYLOs were also radioiodinated prior to SDS-PAGE as to insure that other undetectable apoproteins were not missed (data not shown). In other comparative experiments involving multiple gradient-purified HDLs and serum chylomicrons, only lipoproteins containing apoproteins <14,000
daltons retain their biologic activity. It thus appears that TDC are quantitatively lacking apoproteins in this region and appear to acquire these proteins after their association with the serum and/or vascular endothelial compartment.

Apolipoproteins normally found in this molecular size range of other species, including mice, are characterized as APO C-I, II, III, A II. APO C-I (6,500 daltons) is known to activate lecithin cholesterol acyltransferase, C-II (8,800 daltons) associated with esterification of cholesterol, acts as a co-factor to the activation of endothelial-bound lipoprotein lipase, C-III (8,700 daltons) appears to be an inhibitor of activated lipoprotein lipase, and A-II has no known function to date. To investigate the role of APO C-II activation membrane-bound lipoprotein lipase being responsible for OIF's activity, serum from laboratory mice recently characterized as having a combined lipase deficiency (cld) were evaluated. Animals that are homozygous for the trait are characterized as having inactivated hepatic and lipoprotein lipase (26) and develop massive hyperchylomicronemia (27), and die by 3 days following suckling with triglyceride levels exceeding 20,000 mg/dl (90 to 120 mg/dl being normal). OIF values in these sera usually contained between 20,000 and 40,000 OIF units/ml (data not shown). This finding strongly suggests that one of the major enzymes utilized in fatty acid metabolism is not required for the anti-retroviral effect of OIF.

Thus, it appears from the data presented here that OIF associated with serum CHYLOs are not derived from the intestine, mesenteric lymph nodes, or thoracic duct lymph and do require the
blood vascular compartment for activation. Comparative chemical compositional analysis of TDC and serum CHYLOs reveal little differences excepting the apolipoprotein profiles; however, a 1,200-fold difference in serum CHYLOs OIF activity is noted. Simple lymphatic associated OIF-like blocking factors do not appear to be responsible for this; however, more complex enzymatic removal/activation may be required by the serum and/or vascular endothelium and cannot be ruled out based on these studies. Currently, all these avenues are under investigation. This observation should now provide the means for direct comparisons of active OIF-associated serum CHYLOs to nonactive TDC. This should lead ultimately to identification of the factor, its purification, and a new understanding for the relationships that may exist between retroviruses and circulating lipoproteins. Finally, OIF has been shown to be transferable to human lipoproteins (4) and should its activity include the human retroviruses, its characterization and isolation may provide alternate therapeutic applications.
<table>
<thead>
<tr>
<th>Virus Classification</th>
<th>Virus Classification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant</td>
<td>Recombinant</td>
<td>8</td>
</tr>
<tr>
<td>Recombinant</td>
<td>Recombinant</td>
<td>8</td>
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<tr>
<td>Recombinant</td>
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<td>8</td>
</tr>
<tr>
<td>Recombinant</td>
<td>Recombinant</td>
<td>23</td>
</tr>
<tr>
<td>Recombinant</td>
<td>Recombinant</td>
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</tr>
<tr>
<td>Recombinant</td>
<td>Recombinant</td>
<td>23</td>
</tr>
<tr>
<td>Xenotropic</td>
<td>Xenotropic</td>
<td>5</td>
</tr>
<tr>
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<tr>
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<td>5</td>
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<tr>
<td>Xenopseudotypes</td>
<td>Xenopseudotypes</td>
<td>5</td>
</tr>
<tr>
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<td>Xenopseudotypes</td>
<td>24</td>
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<tr>
<td>Avian</td>
<td>Avian</td>
<td>25</td>
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</table>
Table 3.2. The various sources of OIF are listed. Their chemical compositional analysis was done in a 1000 D microassay system provided by Litton Bionetics, Rockville, MD. The OIF assay is a typical virus neutralization assay in S+L- mouse fibroblast (FG-10) cells and has been previously described elsewhere (5). The OIF units (O.U.) are calculated by the formula $O.U./ml = 0.5 \frac{1}{V^q} (1/dilution)$ where the OIF concentration is related to the virus surviving fraction ($V^q_{no}$) and expressed in 50% inactivating units (5). The values on the left of each data column represent samples from fasted mice and those immediately to the right are values obtained from the same animals (n=4) after oil gavage.

<table>
<thead>
<tr>
<th>Source</th>
<th>Total phospholipid (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Total protein (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>OIF (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole serum</td>
<td>249, 255</td>
<td>135, 142</td>
<td>6500, 7000</td>
<td>100, 150</td>
<td>1,000, 60,000</td>
</tr>
<tr>
<td>Serum CHYLOs</td>
<td>22.6, 28.3</td>
<td>8, 10</td>
<td>12.8, 13.7</td>
<td>99, 200</td>
<td>1,200, 50,000</td>
</tr>
<tr>
<td>Thoracic Duct CHYLOs</td>
<td>-, 33.9</td>
<td>-, 6.0</td>
<td>-, 60.3</td>
<td>-, 97.0</td>
<td>-, 50</td>
</tr>
<tr>
<td>Thoracic Duct Lymph (undil.)</td>
<td>-, 161.2</td>
<td>-, 37</td>
<td>-, 675</td>
<td>-, 633</td>
<td>-, 100</td>
</tr>
<tr>
<td>Intestinal lymph (undil.)</td>
<td>-, 168.4</td>
<td>-, 40</td>
<td>-, 690</td>
<td>-, 650</td>
<td>-, 50</td>
</tr>
</tbody>
</table>
Table 3.3 The Effect of Fasted, Nonheat and Heat-Inactivated Serum on the OIF Activation of TDC

<table>
<thead>
<tr>
<th>Source</th>
<th>OIF (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Whole Serum (nonheat-inactivated)</td>
<td>2,143</td>
</tr>
<tr>
<td>2. Whole Serum (heat-inactivated)</td>
<td>1,99</td>
</tr>
<tr>
<td>3. TDC + Whole Serum (nonheat-inactivated)</td>
<td>825</td>
</tr>
<tr>
<td>4. TDC + Whole Serum (heat-inactivated)</td>
<td>829</td>
</tr>
<tr>
<td>5. Serum CHYLOs + Whole Serum (nonheat-inactivated)</td>
<td>10,350</td>
</tr>
<tr>
<td>6. Serum CHYLOs + Whole Serum (heat-inactivated)</td>
<td>10,100</td>
</tr>
</tbody>
</table>

Equal volumes (100 μl) of TDC and whole heat and nonheat-inactivated serum were mixed together and incubated at 37°C for 90 minutes and tested in the OIF assay. Equivalent concentrations of TDC and serum CHYLOs were added to the sera based on turbidity measurements (absorbance 550) and total phospholipid, protein, triglyceride and cholesterol determinations as mentioned in Table 3.2. The OIF activity of these chemically equivalent preparations were; TDC ≤ 50 O.U./ml and serum CHYLOs = 20,000 O.U./ml.
Fig. 3.1 Microcannulation of the main intestinal lymphatic trunk of the BALB/c mouse. The microcannula is located between the left liver lobe (to the left) and the left kidney (to the right). The glass cannula is completely filled with intestinal lymph and appears white. Lymph was assayed for OIF activity only in samples collected from cannulaus introduced successfully into the lymphatic vessel on the first attempt. The lymph did not clot when collected in this fashion.
Fig. 3.2 Kinetics of OIF in the various body compartments following oil gavage in BALB/c mice (n=4). Serum and thoracic duct chylomicrons were purified by a potassium bromide gradient ultracentrifugation and fractions of d <1.006 g/ml, stained positively with Oil Red O, remained at the origin of a 0.5% agarose gel, and exhibited characteristic apolipoprotein patterns on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (Fig. 3.4). The zero time-point is following an 18-hour fast and just before oil gavage. The whole serum (O–O), purified serum CHYLOs (O–O) and thoracic duct CHYLOs (–) OIF values are plotted. Only whole serum and serum CHYLOs show a steady paralleling increase in activity and reach a peak between 90 and 100 minutes. This correlated with the progressive degree of lipemia (increase in chylomicron fraction) found in the serum. The lower zero time value for serum CHYLOs are due to the 18-hour fast imposed on the animals prior to initiation of the study, and is related to the absence of serum CHYLOs with the bulk of activity being derived from VLDLs.
Kinetics of OIF in Lymph and Blood Following Oil Gavage

Fig. 3.2
Fig. 3.3 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile of various thoracic duct and serum lipoprotein preparations isolated during microcannulation studies in BALB/c mice. Lane 1 demonstrates the apoproteins present in native thoracic duct lymph (100 O.U./ml). Lane 2 demonstrates the apoprotein profile from chylomicrons isolated from the thoracic duct lymph by one potassium bromide gradient centrifugation (d >1.006 g/ml); APO A-IV = 46,000 daltons; APO A-I = 28,000 daltons, and unidentified protein at 12,000 daltons (<50 O.U./ml). Lane 3 represents the apoprotein profiles from thoracic duct chylomicrons isolated by 3 successive potassium bromide gradients (<50 O.U./ml). Lane 4 demonstrates the apoproteins present in serum CHYLOs isolated from one potassium bromide gradient (d<1.006 g/ml) and contains = 10,000 O.U./ml. Lane 2 represents the apoprotein profile from serum CHYLOs after 3 successive potassium bromide gradients; only apoproteins <14,000 daltons were present; however, this lane still contains = 10,000 O.U./ml. Apolipoprotein profiles presented here are consistent with those previously reported in the mouse (28,29).
REFERENCES


15. Reynolds, J.T., and Panem, S. Characterization of antibody to C-type virus antigens isolated from immune complexes in kidneys.


CHAPTER IV

IN VIVO PROTECTION WITH MURINE LIPOPROTEIN-ASSOCIATED RETROVIRAL INACTIVATING FACTOR FOLLOWING RETROVIRAL-INDUCED LEUKEMIA/LYMPHOMA

DEVELOPMENTAL BIOLOGY AND THE EFFECTS OF DIET ON MURINE LIPOPROTEIN-ASSOCIATED RETROVIRAL INACTIVATING FACTOR IN ADULT MICE

INTRODUCTION

Lipoprotein-associated oncornavirus inactivating factor (OIF) has recently been described as existing only within murine members of the subgenus: Mus, species: musculus, domesticus, and cookii (Nara et al., unpublished observations). This lipoprotein-associated factor is found associated with the following serum lipoproteins: chylomicrons (CHYLOs), very low density (VLDLs) and high density lipoproteins (HDLs) (Nara et al., unpublished observations), and inactivates xenotropic (X-), recombinant (RM-) and some polytropic murine leukemia viruses (MuLVs). The factors physical/chemical characterizations have been previously reported (Nara et al., unpublished observations) and are too extensive to list here. Recently, however, the in vitro mechanism of inactivation has been elucidated.
(Nara et al., unpublished observations). OIF was found to bind to both R- and E-MuLV, however, only inactivation of the RM virus occurred. E- and RM-MuLV/OIF complexes were found to bind with equal or greater affinity to the cell membrane as compared to virus alone. No viral lysis was detected by the release of reverse transcriptase or metabolically labeled ribonucleic acids. OIF was also found to inactivate greater than 90% of preadsorbed virus following a 30 to 60 minute incubation and thus appears to exert its biological effects at the host's cell membrane.

It has been previously suggested that OIF is responsible for genomic masking of murine retroviruses in the host (4) and that the absence of directly isolatable RM- and X-MuLVs is also under the influence of this serum factor (5). In earlier studies (6), newborn BALB/c mice were determined to have little OIF in their serum (<10 OIF units/ml) and were found to be susceptible to RM-MuLV (HIX)-induced leukemia lymphoma when injected intraperitoneally. HIX could be isolated directly from serum in these animals in the more terminal stages of disseminated lymphoma which involved the liver, spleen, most lymph nodes, pancreas, and kidneys. These animals were cachectic and found to contain very little OIF in their serum. Serum lipoproteins were not characterized in this study, however, knowing that the factor is associated with CHYLOs, VLDLs and HDLs, most if not all of these lipoprotein classes are severely affected when organ site synthesis and dietary intake are compromised. Also reported in this study, was a direct relationship of increasing age with OIF titers, with adult levels being established after 3 months. There
was no mention of the dietary status of these animals at the time of serum collection. In our earlier report we demonstrated that maximal OIF levels are obtained after post-oil gavage thoracic duct CHYLOs are allowed to associate with the vascular compartment (Nara et al., unpublished observations). Mixing experiments suggest that the vascular endothelium per se or the vascular endothelium of a particular capillary bed organ (i.e., liver, spleen, lung) might be required for activation. Following the observation that chylomicronemia yielded large amounts of OIF, we tested serum from newborn laboratory mice known to develop massive post-suckled chylomicronemia due to an inherited autosomal defect (combined lipoprotein lipase deficiency [CLD]) and usually die by two to three days. The serum from these animals had tremendous levels of the factor (25,000-30,000 O.U./ml). The above observations suggest that OIF is probably present in the neonates, however, the titer may have a wide range of values and appear in a cyclic fashion following suckling. The route of inoculation can be considered important because of the compartmentalization aspects that the factor apparently exhibits and may be considered critical in the earliest stages of viral challenge. Also, OIF's effect on the virus prior to inoculation has never been reported and, as such, the actual effect in the host is not known. It was the intention of these studies to characterize the developmental biology of OIF in the neonate and newborn and to evaluate the in vivo effect of OIF on RM-MuLV (HIX) and E-MuLV (1869) using a HIX viral challenge leukemia/lymphoma model.
MATERIALS AND METHODS

Cells and Virus.

Mouse 3T3FL cells, one of their sarcoma-positive leukemia-negative (S+L-) cloned sublines, FG-10 and feline embryo fibroblasts have already been completely described (7,8). The ecotropic MuLV (1869) used in these experiments and grown in 3T3FL cells were derived from Moloney MuLV cloned by single-focus isolation and endpoint limiting dilution techniques as described previously (8). The poltropic recombinant MuLV(HIX), produced in feline embryo fibroblasts (FEF), were derived from the infection of 3T3FL cells with Moloney MuLV and isolated as described earlier (8). The mouse S+L- fibroblast cell lines (FG-10) were used for all inactivation and binding studies.

Animals and Inoculations.

HIX and 1869 virus was collected as FEF or 3T3FL tissue culture supernates, centrifuged to remove cells and cellular debris, and passed through 0.45 μm filters. This material was then aliquoted and frozen, after which titrations were performed on the lot in S+L- indicator cell lines. Mice used for these experiments were obtained from the animal production facility at the Frederick Cancer Research Facility (Frederick, MD). Groups of pregnant BALB/c mice were synchronized to deliver within one to two days of each other and all animals that had delivered within a 24-hour period were placed in a challenge or control group (n=20). Newborn mice were injected intraperitoneally with approximately $2 \times 10^4$ FITU of either native or OIF
treated (CHYLOs or HDLs) HIX or 1869 MuLV. Virus was diluted 1:1 with either CHYLOs, HDLs or phosphate-buffered saline to a final volume of 100 µl. This material was titered prior to the experiment to assure that >99% of the input virus was inactivated. The control group received cell culture supernate prepared as described above, from the uninfected cell lines. Several of the inoculated mice died within a few days; the cause of death appeared to be associated with maternal aggression and rejection. Animals were weighed, and palpated weekly for spleens and nodes. When moribund, animals were sacrificed, their serum collected, and grossly affected organs were saved for imprint slide preparation, virus extraction, and histopathology. Organs were weighed at necropsy and tumor extracts (approximately 10%) were prepared by the addition of nine volumes of complete medium prior to homogenization in glass grinders.

Other pregnant mice underwent cervical dislocation and near-term fetuses were removed from the uterus and decapitated for collection of blood to be analyzed for lipoprotein characterization and OIF determinations. Also collected were newborn mice which were allowed to suckle and those that were not. Sera from these animals were also collected as previously mentioned.

Agarose Gel Electrophoresis.

Serum lipoproteins were separated and characterized into classes by agarose gel electrophoresis as described by Nobel et al. (10). Briefly, serum to be tested (10 µl) were loaded into 75 x 50 mm 0.5% agarose gels. Following the addition of a bromophenol blue stained albumin solution as a marker, the gels were run at 120 mA per gel in
a 0.05M barbital buffer bath (pH 8.3) for approximately 2-1/2 hours, or until the albumin marker had moved 45 mm into the gel. The gels were then fixed for 30 minutes in a glacial acetic acid/ethanol mixture placed onto standard chromatography paper and dried in a forced air oven for approximately 60 minutes at 80°C. The gels were then stained with Oil Red O to localize the lipoproteins.

**Virus Inactivation of Neutralization Assays.**

Ecotropic Moloney MuLV (1869) and FM-MuLV (HIX) were titrated in FG-10 mouse S+L- cells. Assays for mouse serum-inactivating factor were performed as previously described (3). Briefly, a dilution of heat-inactivated mouse serum was incubated with approximately 200 focus-forming or focus-inducing units of virus FFU or FIU, respectively) for one hour at 25°C, and the resulting mixture was plated on appropriate indicator cells. The relationship in focus reduction by the serum at a given dilution can be expressed as the OIF inactivation units (O.U.) as related to the virus surviving fraction (\(V_n/V_0\)) (2), and calculated by the formula, 

\[ \text{O.U.} = (0.5)(1/V_n/V_0)(1/\text{dilution of serum}) \]

Levels of OIF <50 O.U./ml are considered nonsignificant. This value was based on nonspecific neutralization of both ecotropic and recombinant MuLVs with artificial lipoprotein preparations of similar concentrations.

**Virus Isolation.**

Serum and tumor extracts were assayed for virus isolation and characterization. Tumor extracts were used in an infectious cell center assay on the previously mentioned S+L- cell lines. Details of the assay have previously been published (11). Briefly, lymphoid
cells from involved spleens were tested by using dilutions of washed cells \((10^6 - 10^3 \text{ per dish of mouse or cat S+L- cells})\) and determining the proportion of cells yielding virus in either test system. Limiting dilution foci selection was used as a means for selecting a single clone for virus purification and identification.

**Dietary Studies.**

Adult BALB/c mice were obtained as previously mentioned from the Frederick animal production facility. Animals were placed into groups of 20 animals with equal numbers of male and females present. All animals were fasted 18 hours prior to receiving gastric gavage of olive oil. Prior to this, all animals were bled from the medial canthus of the eye and the blood pooled for OIF determinations, serum electrophoresis, blood lipoprotein chemistry, and purification of lipoprotein constituents by potassium gradient ultracentrifugation. Animals were allowed to drink ad libitum; however, no solid food was allowed over the course of the experimental period (6 days), at which time they were terminally exsanguinated. Daily blood samples were taken and characterized as previously mentioned.

**Lipoprotein Purification.**

The blood samples were collected and pooled into 30 ml glass conical centrifuge tubes and allowed to clot at room temperature. Serum was separated, heat-inactivated and prepared for lipoprotein isolation as described (9). Briefly, a 4 ml aliquot of serum was adjusted to \(d=1.21 \text{ g/ml}\) by the addition of solid potassium bromide salt (KBr, 0.325 g/ml of serum) in SW-41 centrifuge tubes. Continuous gradients were formed at ambient temperature with the use of a
Haake Buchler Autodensiflow II (Buchler Instruments, Searle Analytical, Inc., Fort Lee, NJ) gradient maker using KBr salt solutions of d=1.063 and 1.019 at a speed of 1.0 ml/min. Finally, the tubes were topped off with 2.0-3.0 ml of d=1.006 g/ml KBr solution containing 1.0 mM EDTA and 1% penicillin/neomycin/streptomycin. The samples were then centrifuged at 36,000 rpm for 18 hours at 15°C in a Beckman LS Model 350 ultracentrifuge and fractionated into 0.5 ml fractions with the aid of a Gilsen micro-fraction collector. The fractions were then checked for purity by agarose gel electrophoresis and chemical composition analysis; phospholipid, protein, triglyceride, total cholesterol and free cholesterol (1000D microassay, Litton Bionetics, Rockville, MD). Fractions containing the major lipoprotein classes of similar density classes were pooled and dialyzed in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA; exclusion limit ca. 3,500) for 3X 24 hours at 4°C in phosphate buffered saline.
RESULTS

Characterization of Age-Related OIF Titers.

The developmental biology of OIF in near-term and neonatal mice are represented in Figs. 4.1 and 4.2 and Table 4.1. Serum taken from 21 to 22 gestational day near-term fetuses and newborn nonsuckled neonates had no detectable OIF titers (Table 4.1) and the agarose gel lipoprotein profiles are presented in Fig. 4.2. Lane 3 (near-term serum) and lane 4 (presuckled serum) are similar in pattern and are compared to their mother's serum (lane 2). Profound alterations occur following ingestion of colostrum and are reflected by the loss of an intensely oil red O staining band in the pre-beta region (lane 5). This band corresponds to low density lipoproteins (LDL) (based on migration properties in these gels), however, the mouse is considered a species that has a relatively low level of this lipoprotein species (12,13), and has been suggested that cholesterol transport in this species is mediated by HDLs (12,13). This band stains with the intensity comparable to HDLs found in the alpha region of the maternal serum. No chylomicrons are seen at the origin and very little staining is seen throughout the beta-alpha region, indicating the diminished level of very low density lipoproteins (VLDL) (lanes 3 and 4). Serum collected from pooled, post-suckled neonates (lane 5) exhibits a ten to 50-fold increase in OIF activity (Table 4.1) and is reflected by a significant alteration in the lipoprotein profile. The intensely staining pre-beta band seen in lane 4 is completely gone and replaced by a diffusely stained band
(VLDL) extending from beta to alpha regions in the gel. Also, chylomicrons were seen as the arching band found at the origin (lane 5). Animals that had previously suckled and were denied milk (2-4 hours) were found to return to an OIF-negative status (data not shown). These animals also failed to develop an alpha migrating HDL band as determined by agarose gel electrophoresis (data not shown). The only significant change noted in the serum of older animals (2-9 weeks) was the appearance of the prominent HDL band in 18 hours fasted sera (similar to lane 2) and corresponded to more stable OIF levels during periods of fasting.

In order to rule out that OIF was present in the colostrum and passively absorbed by the suckling neonate, colostrum from these same and other lactating mice were pooled, heat-inactivated and tested for its ability to inactivate RM-MuLV (Table 4.1). No inactivating activity was found. Another rule-out considered that the factor could be passively absorbed and later activated by either the association with the neonatal stomach prior to absorption, or that activation may occur in the vascular compartment. No activity was found in digested milk taken from the mouse stomachs, nor was any activity found from mixing fresh neonatal whole blood with digested milk from the stomach (Table 4.1). Also, peritoneal fluid was collected from twenty-five normal adult mice by lavage with physiologic saline and concentrated to a final volume of 200 microliters (represents approximately 25 X concentrate) and tested for OIF activity (Table 4.1). No OIF activity was detected in this body compartment. This was considered important to know in light of an intraperitoneal viral
challenge in the neonate. The average volume of peritoneal fluid that could be collected by microcapillary tube directly from the peritoneum was 25-50 μl. Eighteen hour fasted serum from these animals always contained between 1,000 and 5,000 O.U./ml. **Inoculation of Mice with RM- and E-MuLV**

**Clinical, Gross, and Histopathology.**

No immediate clinical responses were seen with all surviving animals up to five weeks of age. Variable neonatal death related to maternal aggression was seen in some animals receiving intraperitoneal inocula. By five and eight weeks of age, animals in litters receiving HIX or 1869 MuLV, respectively (Group 1 of Tables 4.2 and 4.3), were described as unthrifty with a rough hair coat and generally weighed less as a group per week. Clinical signs were similar for both virus inocula and, as such, only the HIX-derived disease will be described in detail.

Initial signs developed first, however, in HIX-challenged BALB/c mice as detailed in Table 4.2. Palpable spleens were first observed in seven week old animals. The disease progressed rapidly with palpable axillary, inguinal and mesenteric lymph nodes. Animals died or were sacrificed when moribund on the average of twelve weeks after inoculation. Only those animals which had histopathological diagnosis of lymphoma were scored as positive. The actual tumor incidence for both E- and RM-MuLV challenged mice approached 100%. Representative histopathology is depicted in Figs. 4.3-4.6. All animals with lymphoma had large to enormous (4 gm) spleens. Mesenteric nodes were usually quite large (1-2.5 cm in diameter). Thymus
was moderately enlarged (2-3X) in five of fourteen animals receiving 1869 and eight of seventeen animals receiving HIX.

The livers were generally involved and consisted of periportal and centrolobular sinusoidal infiltration of large pale undifferentiated lymphoblasts. Numerous, more differentiated, smaller, basophilic lymphocytes were noted in the central veins and peripheral blood smears. Kidneys and pancreas were also uniformly involved but to variable extend and were characterized by focal areas of large pale undifferentiated lymphoblasts effacing normal architecture in all animals.

All animals receiving 1869 incubated with either CHYLOs or HIX (groups 3 and 4, Table 4.3) also developed lymphocytic lymphoma and could not be distinguished from virus controls (group 1).

Animals receiving HIX incubated with OIF containing serum lipoprotein fractions, CHYLOs and HDLs, respectively, were completely protected from the development of HIX-induced lymphoma and could not be distinguished from media controls (group 2). No other spontaneous tumors were detected in this study which involved a total of 160 animals, however, complete necropsies were not always performed.

**Virus Isolation from 1869 and HIX Mouse Tumors.**

Five tumors from each group of HIX and inoculated animals were examined in detail for the presence of virus and its properties. As outlined in Table 4.2, each individual tumor extract from the mice had high titers of infectious virus for both mouse and cat S+L- assay systems. Titers were somewhat higher in tumor extracts from HIX-derived tumors in mouse cells, indicating that possibly ecotropic
virus was also present. Because a virus stock with amphotropic virus properties could also reflect a mixture of eco- and xenotropic viruses, several experiments were performed to characterize the virus stock. Initially, it was clear that the nature of foci at limiting tumor-derived dilutions in the S+L- 81 cat cell clone was characteristic of HIX with typical polykaryocyte areas. To rule out a significant pure ecotropic virus component tumor, extracts were treated with high titered serum CHYLOs (10,000 O.U./ml) which could inactivate a standard FEF cell-derived HIX or B-MuX MuLVs to a $V_n/V_o=0.001$ of the original titer. The same serum did not inactivate 1869 MuLV. No ecotropic virus was detected, in that all tumor-derived virus capable of forming foci in mouse S+L- cells was inactivated by OIF containing CHYLOs. To determine definitively that individual units of tumor-derived virus was amphotropic, we picked limiting dilution foci from mouse S+L- cells. Virus progeny from all single foci was able to grow in both mouse and cat cells.

Ecotropic (1869) MuLV-derived tumors were also evaluated for the presence and characterization of virus type and are presented in Table 4.3. Tumor extracts yielded large amounts of virus that would infect and transform only mouse cells and were resistant to the inactivating characteristics of OIF. Virus could be isolated from the blood of these animals generally any time after approximately sixty days post-inoculation and were capable of transforming only mouse cells. Five tumors were tested from groups 1, 3, and 4 for the presence of amphotropic virus by infectious cell center assays. Lymphoid cells from the spleen, liver and thymus were tested by using
dilutions of washed cells (10^{-6} - 10^{-3} per dish of mouse or cat S+L-cells) and determining the proportion of cells yielding virus growth in cells of tested organs. Amphotropic MuLV-positive cells were detected in all terminal organs tested and found to be sensitive to OIF (data not shown).

**Dietary Study.**

The effects of fasting followed by fat ingestion on OIF levels are presented in Fig. 4.7 and Table 4.4. A significant elevation in OIF levels occurs within 60 minutes (17,150 O.U./ml) after the introduction of dietary lipid. Maximal levels of OIF are seen by 90 minutes and reach levels of 60,000 O.U./ml in some experiments. This level was elevated for up to 240 minutes post-gavage and exhibited a half-life of approximately 12 hours. The OIF levels are reduced slowly over the first day (Fig. 4.7) and fall rapidly (3-fold reduction) by the second day, after which the slope of OIF decay is decreased out to day six (450 O.U./ml in pooled serum).

More specific characterizations of this dietary-related OIF response are presented in Table 4.4. The majority of oil-induced OIF activity was directly related to the serum chylomicron compartment (d=<1.006 g/ml) and was accompanied by an increase in total protein and triglyceride levels of approximately 5-fold when compared to protein values from similar gradients taken from 18 hour fasted serum (Table 4.4). Also, noted was a 7-fold increase in OIF values obtained from the d=1.006-1.063 g/ml range and were found to be due to CHYLO remnants and VLDLs (data not shown). HDLs exhibited a 7-fold increase in OIF levels and were associated with small increases in
all measured chemical values. By one day post-gavage, the CHYLO-associated OIF levels were 10,625 O.U./ml and again corresponded to reduction in both total protein and triglycerides of approximately 5-fold. Only a slight increase in OIF-associated HDL activity is seen at this time, however, by two days post-gavage, a 3-fold increase in OIF-associated HDLs were noted and may be associated with a 2-fold increase in the total protein of these fractions. OIF-associated CHYLOs at this time are reduced to 410 O.U./ml and are lost by 5 days. The HDLs were found to contain the majority of OIF from the 2nd day of fasting until the termination of the experiment.
DISCUSSION

The discovery of OIF at supra-normal levels previously reported in combined lipoprotein lipase-deficient neonates (Nara et al., unpublished observations) indicates that neonatal mice have the factor at an earlier age than was previously considered (6). It also suggested that suckling followed by elevations in serum chylomicrons may demonstrate significant quantities of OIF. The results reported here confirm that serum from developing mouse embryos lack any OIF activity in utero and post-natally, or until they suckle. Also, the possibility of obtaining the factor as a passive mechanism seems unlikely for the following reasons. First, no activity was found in the mother's milk; secondly, no activity was associated with digested milk taken from the neonatal stomach - suggesting that activation does not occur prior to absorption. Thirdly, no activity was found when digested milk was exposed for 60 minutes at 37°C to whole blood from the neonates. These findings suggest that passive absorption of a factor from milk which is subsequently activated upon by other factor(s) in the blood is not the likely mechanism for elevated OIF levels in post-suckled serum. However, because of the inability to cannulate neonatal thoracic ducts and collect thoracic duct chylomicrons as previously reported (Nara et al., unpublished observations), we could not rule out the possibility that inactive factor could be passively or actively absorbed, incorporated in chylomicrons in the enterocytes and be biologically active upon entering the lymphatics, prior to its introduction into the circulation. Thus, it
appears that neonatal mice have cyclic periods of OIF (directly related to post-suckled lipemia) present in their serum during the first few days of life and therefore probably had some levels during the viral challenge.

It seems apparent from the neonatal studies, that 24 hour old animals used in the viral challenge should have some levels of OIF present. In a parallel study done at the time of inoculation, OIF levels were determined in sera from a comparable pool of 24 hour old neonates in various stages of post-suckled lipemia. The results indicate that the pool contained approximately 250 O.U./ml and contained some sera with as much as 500 O.U./ml and other with <50 O.U./ml (data not shown). This finding along with the absence of OIF levels in peritoneal fluid suggests that the route of viral inoculation may be considered significant when challenging these animals. It is not surprising that these animals become infected following an intraperitoneal challenge due to the tissue potential available to the virus upon entry into the host and in an environment lacking OIF.

A preliminary finding, based on a small number of animals (n=5/6), was the uniform susceptibility of animals (neonates) to subcutaneous challenge. Many animals died due to the maternal rejection and aggression associated with subcutaneous hematomas purposely induced in the challenges. A study that was not done, however, which would serve as conclusive proof that the route of inoculation may be significant, would be to give the inoculum intravenously, or repeat the previous protocol with sera collected from post-suckled lipemic
animals, thus assuring the challenge virus to endogenous OIF and allow for the real determinant of protection in these animals.

The uniform protection of RM-MuLV and the uniform susceptibility of E-MuLV of all animals receiving virus preincubated with OIF containing lipoprotein fractions suggests that the in vitro inactivating activity previously characterized, is very relevant to the in vivo biologic activity noted in these experiments. As reported earlier (Nara et al., unpublished observations), the in vitro aspect of inactivation seems to involve the cell membrane and strongly suggests that inactivation would involve the host's cells in vivo. In this case, however, we could not determine if the RM-MuLV was inactivated prior to the inoculation into the animals. The extreme sensitivity of this virus to temperature and handling precludes any post-incubation manipulations directed at separation of these complexes.

The dietary studies done on the adult BALB/c mice indicate again that enhanced OIF levels can be generated following the ingestion of high levels of dietary lipid. This activity was conclusively shown in this study to be associated with the serum CHYLOs. An interesting finding was the 5-fold increases noted for both total protein and triglyceride components in those high titered OIF CHYLOs isolated after oil gavage. It has been reported previously that both the lipid and protein are required for maximal OIF activity. Also, dietary lipid preparations such as Lipomule (Upjohn Co.) is found to have some (<=50 O.U./ml) nonspecific inactivating characteristics as reported previously (Nara et al., unpublished observations). This
dietary manipulation leading to elevated and isolatable OIF in a serum lipoprotein compartment such as CHYLOs should allow for the characterization and purification of the factor. The fasting studies serve to confirm the distribution and dynamics of OIF activity in the previously accepted lipoprotein classes and again confirms the dependence of dietary and endogenous lipoprotein metabolism on the levels of circulating OIF. This finding also confirms that any factors which compromise normal levels of either CHYLOs, VLDLs and HDLs could allow for OIF titers to fall below certain (as yet undetermined) levels which then allow for the expression of recombinant and/or xenotropic MuLV in vivo, a situation reported to occur in the terminal stages of RM-MuLV-induced lymphocytic lymphoma and seen in the present study.

In conclusion, it appears that animals having significant OIF levels can be infected by RM-MuLV if entry into the host is by the OIF-negative compartments. OIF is directly related and influenced by dietary perturbations and appears to be capable of complete inactivation of RM-MuLV in vivo following incubation in vitro with high titered OIF containing serum lipoprotein fractions (i.e., CHYLOs and HDLs).
SUMMARY

Isolated chylomicrons (CHYLOs) and high density lipoproteins (HDLs) known to contain a lipoprotein-associated retroviral-inactivating factor (OIF) were found to be capable of completely protecting mice from the development of recombinant murine retroviral (RM-MuLV)-induced leukemic lymphoma. Infectious virus was incubated with purified serum CHYLOs and/or HDLs prior to intraperitoneal injection of neonatal mice. Ecotropic MuLV incubated in parallel fashion with these same OIF fractions failed to protect animals from the development of viral-induced lymphoma.

Also, the developmental biology of the factor in BALB/c mice was investigated. Near-term fetuses and neonatal pre-suckled animals have no factor present in their serum. However, as much as a 10-fold increase in OIF is generated following the ingestion of milk and directly associated with the serum CHYLOs. Post-suckled neonates kept from suckling contain OIF-negative serums. The development of fasted levels of OIF appears to coincide with the development of alpha migrating HDLs. The ingestion of dietary lipid in adult mice causes a tremendous increase in the level of circulating OIF associated with the serum CHYLOs. These levels are found to decline 10-fold during prolonged fasting (six days), and by two days. HDLs are found to contain most of the anti-viral activity.
Table 4.1 Developmental Biology and Compartmentation of OIF in BALB/c Mice.

<table>
<thead>
<tr>
<th>OIF Source</th>
<th>OIF Levels (O.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-sectioned sera</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Neonatal sera/nonsuckled</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Neonatal sera/suckled (full stomach)</td>
<td>500</td>
</tr>
<tr>
<td>Neonate sera/post-suckled (empty stomach)</td>
<td>≤50</td>
</tr>
<tr>
<td>Mother sera</td>
<td>1,300</td>
</tr>
<tr>
<td>Mother's milk (undigested)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Peritoneal fluid only</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Milk (undigested) + nonsuckled whole blood</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Milk (digested) only</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>
### Table 4.2 In Vivo OIF/EM-MuLV Challenge Study in Newborn BALB/c Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals</th>
<th>Animals*</th>
<th>Time of onset+</th>
<th>Presence of HIX in tumor (titer in extract)</th>
<th>Presence of ecotropic virus in tumors</th>
<th>OIF levels†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c mice newborns inoculated</td>
<td>20</td>
<td>3</td>
<td>13/17</td>
<td>7-12 weeks</td>
<td>5/5 (1.0 x 10^6 FL U/ml)</td>
<td>100</td>
</tr>
<tr>
<td>Diffuse lymphoblastic leukemia lymphoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II n=20</td>
<td>20</td>
<td>4</td>
<td>0/16</td>
<td>--</td>
<td>0/5</td>
<td>2500</td>
</tr>
<tr>
<td>(Media)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III n=20</td>
<td>20</td>
<td>7</td>
<td>0/13</td>
<td>--</td>
<td>0/5</td>
<td>3000</td>
</tr>
<tr>
<td>(CHYLOs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5, 0/5</td>
<td></td>
</tr>
<tr>
<td>Group IV n=20</td>
<td>20</td>
<td>5</td>
<td>0/15</td>
<td>--</td>
<td>0/5</td>
<td>2500</td>
</tr>
<tr>
<td>(HDLs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5, 0/5</td>
<td></td>
</tr>
</tbody>
</table>

*Death usually due to maternal rejection and/or infanticide.

†Decreased weight gain, rough hair coat, lethargy, hepatosplenomegaly, lymphadenopathy, thymomegaly.

‡OIF levels determined in pooled terminal bleeds.
Table 4.3 **In Vivo OIF/E-MuLV Challenge Study in Newborn BALB/c Mice**

<table>
<thead>
<tr>
<th>BALB/c mice inoculated</th>
<th>Animals* dying acutely</th>
<th>Tumor incidence</th>
<th>Time of onset+ for clinical signs after inoculation</th>
<th>Presence of HIX in tumor (titer in extract)</th>
<th>Presence of ecotropic virus in tumors</th>
<th>OIF levels†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Virus)</td>
<td>n=20 4</td>
<td>10/14</td>
<td>19-16 weeks</td>
<td>Diffuse lymphoblastic leukemia</td>
<td>3/5 RM-MuLV (2.0 x 10⁴ FIU/ml)</td>
<td>5/5 E-MuLV (7.4 x 10⁵ FIU/ml)</td>
</tr>
<tr>
<td>Group II (Media)</td>
<td>n=20 5</td>
<td>0/15</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Group III (CHYLOs)</td>
<td>n=20 3</td>
<td>14/17</td>
<td>10-16 weeks</td>
<td>Same as Group I</td>
<td>4/5 RM-MuLV (4/5 x 10⁴ FIU/ml)</td>
<td>5/5 E-MuLV (4.5 x 10⁴ FIU/ml)</td>
</tr>
<tr>
<td>Group IV (HDLs)</td>
<td>n=20 7</td>
<td>10/13</td>
<td>10-16 weeks</td>
<td>Same as Group I</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* Death usually due to maternal rejection and/or infanticide.
+ Decreased weight gain, rough hair coat, lethargy, hepatosplenomegaly, lymphadenopathy, thymomegaly.
† OIF levels determined in pooled terminal bleeds.
Table 4.4 The Effect of Fasting on OIF Levels on Lipoprotein Chemistry of Purified Serum Lipoprotein Fractions

<table>
<thead>
<tr>
<th>Description</th>
<th>Total proteins (mg/dl)</th>
<th>Total phospholipid (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>OIF values (O.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Fast 18 hr) d&lt;1.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1 mg/dl</td>
<td>21.1</td>
<td>2</td>
<td>18</td>
<td>1,210</td>
</tr>
<tr>
<td>d=1.006-1.063</td>
<td>1.1 mg/dl</td>
<td>15.5</td>
<td>2</td>
<td>3</td>
<td>80</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>0.2 g</td>
<td>137</td>
<td>96</td>
<td>18</td>
<td>1,150</td>
</tr>
<tr>
<td>d&gt;1.21</td>
<td>2.6 g/dl</td>
<td>41</td>
<td>4</td>
<td>30</td>
<td>&lt;50</td>
</tr>
<tr>
<td>(60' post-oil) d&lt;1.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.7 mg/dl</td>
<td>22.6</td>
<td>8</td>
<td>99</td>
<td>17,500</td>
</tr>
<tr>
<td>d=1.006-1.063</td>
<td>1.1 mg/dl</td>
<td>21.2</td>
<td>0</td>
<td>1.0</td>
<td>615</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>0.20 g/dl</td>
<td>173</td>
<td>113</td>
<td>29</td>
<td>3,142</td>
</tr>
<tr>
<td>d&gt;1.21</td>
<td>1.69 g/dl</td>
<td>48.1</td>
<td>0</td>
<td>42</td>
<td>&lt;50</td>
</tr>
<tr>
<td>(Fast 1st day) d&lt;1.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 mg/dl</td>
<td>20.2</td>
<td>3</td>
<td>17</td>
<td>10,625</td>
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<tr>
<td>d=1.006-1.063</td>
<td>1.2 mg/dl</td>
<td>14.7</td>
<td>2</td>
<td>3</td>
<td>400</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>0.24 g/dl</td>
<td>140</td>
<td>92</td>
<td>16</td>
<td>4,210</td>
</tr>
<tr>
<td>d&gt;1.21</td>
<td>2.7 g/dl</td>
<td>42</td>
<td>5</td>
<td>32</td>
<td>&lt;50</td>
</tr>
<tr>
<td>(Fast 2nd day) d&lt;1.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9 mg/dl</td>
<td>15.5</td>
<td>0</td>
<td>3</td>
<td>431</td>
</tr>
<tr>
<td>d=1.006-1.063</td>
<td>5.7 mg/dl</td>
<td>14.1</td>
<td>0</td>
<td>4</td>
<td>310</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>0.4 g/dl</td>
<td>197.9</td>
<td>126</td>
<td>24</td>
<td>13,000</td>
</tr>
<tr>
<td>d&gt;1.21</td>
<td>3.3 g/dl</td>
<td>59.4</td>
<td>6</td>
<td>1</td>
<td>&lt;50</td>
</tr>
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</table>
Table 4.4 (Continued)

<table>
<thead>
<tr>
<th>Description</th>
<th>Total proteins</th>
<th>Total phospholipid (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>OIF values (O.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Fast 3rd day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d&lt;1.006a</td>
<td>0.6 mg/dl</td>
<td>14.1</td>
<td>0</td>
<td>2</td>
<td>410</td>
</tr>
<tr>
<td>d=1.006-1.063</td>
<td>1.3 mg/dl</td>
<td>9.9</td>
<td>0</td>
<td>3</td>
<td>170</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>0.2 g</td>
<td>132</td>
<td>90</td>
<td>20</td>
<td>2,500</td>
</tr>
<tr>
<td>d&gt;1.21</td>
<td>2.6 g/dl</td>
<td>65</td>
<td>13</td>
<td>26</td>
<td>&lt;50</td>
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<td>(Fast 4th day)</td>
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<td></td>
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<td></td>
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<tr>
<td>d&lt;1.006</td>
<td>0.7 mg/dl</td>
<td>13.2</td>
<td>0</td>
<td>1</td>
<td>254</td>
</tr>
<tr>
<td>d=1.006-1.063</td>
<td>1.0 mg/dl</td>
<td>9.7</td>
<td>0</td>
<td>3</td>
<td>115</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>0.18 g/dl</td>
<td>130</td>
<td>85</td>
<td>19</td>
<td>1,580</td>
</tr>
<tr>
<td>d&gt;1.21</td>
<td>2.5 g/dl</td>
<td>62</td>
<td>10</td>
<td>24</td>
<td>&lt;50</td>
</tr>
<tr>
<td>(Fast 5th day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d&lt;1.006</td>
<td>4.2 mg/dl</td>
<td>9</td>
<td>0</td>
<td>1</td>
<td>&lt;50</td>
</tr>
<tr>
<td>d=1.006-1.063</td>
<td>1.8 mg/dl</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>0.12 g/dl</td>
<td>50</td>
<td>42</td>
<td>10</td>
<td>1,200</td>
</tr>
<tr>
<td>d&gt;1.21</td>
<td>2.6 g/dl</td>
<td>61</td>
<td>24</td>
<td>27</td>
<td>&lt;50</td>
</tr>
<tr>
<td>(Fast 6th day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d&lt;1.006</td>
<td>10.9 mg/dl</td>
<td>7.1</td>
<td>0</td>
<td>1</td>
<td>&lt;50</td>
</tr>
<tr>
<td>d=1.006-1.063</td>
<td>2.1 mg/dl</td>
<td>1.4</td>
<td>0</td>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>0.1 g/dl</td>
<td>31.3</td>
<td>24</td>
<td>5</td>
<td>510</td>
</tr>
<tr>
<td>d&gt;1.21</td>
<td>2.9 g/dl</td>
<td>60.8</td>
<td>33</td>
<td>31</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

aRepresents the specific densities (g/ml) of fractions collected from potassium bromide gradients for various lipoprotein class isolation and characterization.
Fig. 4.1A Serum lipoprotein profiles of neonatal BALB/c mice as determined by 0.5% agarose gel electrophoresis and stained with Oil Red O. Lane 1, represents the pre-stained albumin marker used as a protein migration marker. Lane 2, represents serum from a fasted mother mouse. Lane 3, represents sera taken from a pool (n=8) of 21 day old c-sectioned fetuses. Lane 4, are sera derived also from a pool (n=8) of 12 hour old, pre-suckled neonates. Lane 5, represents sera taken from a pool (n=8) of 12 hour old suckled neonates.
Fig. 4.1B Serum lipoprotein profiles of neonatal BALB/c mice as determined by 0.5% agarose gel electrophoresis and stained with Coomassie blue. Lane 1, represents the pre-stained albumin marker. Lane 2, represents sera taken from a pool (n=8) of 21 day old c-sectioned fetuses. Lane 3, are sera derived also from a pool (n=8) of 12 hour old, pre-suckled neonates. Lane 4, represents sera taken from a pool (n=8) of 12 hour old suckled neonates. Lane 5, represents serum from a fasted mother mouse.
Fig. 4.2 Developmental biology of OIF in the BALB/c mouse. First time-point represents the OIF levels in 21 day old c-sectioned fetuses. The second time-point was taken from 12 hour old pre-suckled neonates.
Age Related OIF Titers of:
In Utero, Neonatal, Juvenile BALB/c Mice

![Graph showing Age Related OIF Titers of In Utero, Neonatal, Juvenile BALB/c Mice.](#)

Fig. 4.2
Fig. 4.3 Renal parenchymal effacement by infiltrating lymphoblastic cells. Isolated arteriole, renal tubules, and glomerulus can be seen. (2.5 X)
Fig. 4.4  HIX inoculated BALB/c liver. Hepatic effacement with sinusoidal infiltration and isolation of hepatic cords by tumor cells. (10X)
Fig. 4.4
Fig. 4.5 Spleen of a BALB/c mouse. Diffuse replacement of normal architecture by lymphoblastic cells. (10X)
Fig. 4.6 High magnification of tumor cells found in the mesenteric lymph node. Large, pale, undifferentiated lymphoblasts with a characteristic abnormal mitotic figure located centrally. (45X)
Fig. 4.6
Fig. 4.7 Dietary effects of OIF levels of BALB/c mice. The first time-point (-1) represents sera taken from non-fasted (n=6) adult mice. The second time-point (0) represents the OIF levels from (n=6) animals following an 18 hour fast and just prior to oil gavage.
Effect of Diet/Fasting on OIF Levels in Mice

Fig. 4.7
REFERENCES


CHAPTER V

STUDIES ON THE TRANSFER OF MURINE LIPOPROTEIN ASSOCIATED RETROVIRAL INACTIVATING FACTOR TO THORACIC DUCT CHYLOMICRONS.

I. ACTIVITY ASSOCIATED WITH TRANSFER OF LOW MOLECULAR WEIGHT PROTEIN(S)

INTRODUCTION

All mouse strains of Mus: musculus, domesticus contain inherited (endogenous) genes for a class of mouse type C viruses (MuLVs) which has an unusual host range. Although present in the genes of mice, these viruses are unable to reinfect mouse cells of inbred laboratory strains but have the capability of infecting cells of a wide variety of mammalian (including the cells of wild mice and avian species) and avian origin. This special characteristic of these viruses has been called xenotropism (1-3). The xenotropic (X-tropic) viruses, which are uniquely susceptible to the lipoprotein associated oncornavirus inactivating factor (OIF), differ from the endogenous mouse ecotropic viruses, in that they are also inherited in the genes of many mouse strains but can reinfect mouse cells (4). One final class of MuLV that is inactivated by this factor are the recombinants (RM) which have been shown to be recombinations of endogenous xenotropic and
ecotropic MuLV, especially in the envelope sequenced (5). The ecotropic as well as the RM-MuLV viruses have been implicated in the generation of murine leukemia/lymphoma in this species. The role of the mouse X-tropic viruses is unknown.

We reported earlier (Nara et al., unpublished observations) that a correlation appears to exist between the presence of endogenous X-tropic MuLV sequences and the presence of OIF in the serum of all laboratory-derived mice tested to date. Also noted in our study, was preliminary evidence that the factor could be inherited in simple mendelian fashion and may represent the emergence of a resistance gene.

We have also defined the lipoprotein (LP) classes normally found in mouse serum which specifically inactivates the mouse endogenous X-tropic and RM-MuLVs. These LPs have no activity against the ecotropic class of endogenous MuLVs (5-7; Nara et al., unpublished observations). The neutralizing activity as reported by others (6) was found primarily in the triglyceride-rich LPs (chylomicrons [CHYLOs], very low density lipoprotein [VLDLs], and high density lipoproteins [HDLs]). Only very low titers were found with low density lipoprotein (LDLs). Levels of OIF are influenced by diet. Mice given dietary lipid by gavage needle can increase their levels by 20- to 30-fold which is primarily associated with serum chylomicrons (6; Nara et al., unpublished observations) and appears to require the vascular compartment for activation (Nara et al., unpublished observations). Also, thoracic duct chylomicrons (TDCs) were found to be devoid of OIF activity (Nara et al., unpublished
observations) and have thus, provided an important means of studying the de novo activation of this factor.

Earlier reports by Levy, et al. (6) indicated that the factor could be transferred to artificial synthetic ordered lipids, LPs from OIF negative mice and to human LPs (7). Also reported in these studies was the ability for the factor to back transfer to other OIF negative LPs. These studies imply the transfer of a class of apolipoproteins (C's) as the ultimate determinant of activity, however, no biochemical or physical evidence of this protein transfer was presented. This evidence has been difficult to demonstrate previously due to the similarity of LPs apolipoprotein profiles found in similar and unrelated species (8). We reported earlier of the isolation of TDCs which are devoid of OIF activity and represent an in vivo precursor to serum CHYLOs (high OIF containing LPs) with the added benefit of a dissimilar apolipoprotein profile (Nara et al., unpublished observations).

It was the intention of this study to utilize the TDCs for transfer experiments with the hope of identifying the responsible protein(s).
MATERIALS AND METHODS

Cells and Viruses.

Mouse 3T3FL cells, one of their sarcoma-positive leukemia-negative (S+L-) cloned sublines, FG-10 and feline embryo fibroblasts have already been completely described (9,10). The ecotropic MuLV (1869) used in these experiments and grown in 3T3FL cells were derived from Moloney MuLV cloned by single focus isolation and endpoint limiting dilution techniques as described previously (10). The polytropic recombinant MuLV (HIX), produced in feline embryo fibroblasts (FEF), were derived from the infection of 3T3FL cells with Moloney MuLV and isolated as described earlier (10). The mouse (S+L-) fibroblast cell lines (FG-10) were used for all inactivation and binding studies.

Animals and Serum Collection.

Inbred mature male and female BALB/c mice (obtained from the animal production facility, Frederick Cancer Research Facility, Frederick, MD) provided the serum source for these experiments. The animals were maintained on Purina Mouse Chow and water ad libitum. Prior to exsanguination, all mice were fasted overnight. Of these, some animals were given olive oil via stomach tube 90 minutes prior to exsanguination while other animals were bled after the fasting period. All animals were anesthetized and blood collected following jugular transection. Blood from cat, rat, goat, and rabbit were collected as just described.
Thoracic Duct Cannulations.

Large volumes of TDCs were obtained from chronic cannulations in BALB/c mice as described by Boak et al. (11). Briefly, the animal was given concentrated dietary lipid (Lipomule, The Upjohn Co., Kalamazoo, MI) by gavage needle and allowed 60 minutes to digest and begin absorption of the triglyceride into lymphatics. This material causes the intestinal lymphatics as well as the thoracic duct to appear opaque white and allow for immediate localization and cannulation. The animals were then anesthetized (Metofane) and the abdomen opened by an incision following the lateral border of the left quadratus lumborum muscle. The left kidney is mobilized and retracted medially which exposes the duct embedded in retroperitoneal fat which has to be carefully dissected away. Cannulations are made easier by the aid of a variable magnifying binocular dissecting microscope. The cannula is passed through the flank of the animal by means of a trocar, and the beveled end is inserted directly into the duct with a quick stab. Immediately, a drop of methyl 2-cyanocrylate (Krazy Glue Inc., Itasca, IL, 60143) is placed onto the junction of the cannula and lymphatic trunk. Afterward the dissected retroperitoneal fat is repositioned over the cannula and another small drop (0.005 ml) of glue is applied. This serves to anchor the cannula and prevent premature tearing upon recovery of the animal. Also, the cannula is glued at the junction of the abdominal wall. Lymph is collected from the immobilized animals for 18 to 24 hours and would average 15-20 ml.
Lymph from similarly cannulated C57BL/6J-CBA mice was graciously provided by Dr. Johnathan Sprent for comparison to the BALB/c mouse system.

Serum and Thoracic Duct Lipoprotein Isolation and Purification.

All lymph and serum was heat-inactivated and prepared for LP isolation as described by Redgrave et al. (11). Briefly, a 4 ml aliquot of serum or lymph was adjusted to d=1.21 by the addition of solid potassium bromide salt (KBr, 0.325 g/ml of serum) in SW-41 centrifuge tubes. Continuous gradients were formed at ambient temperature with the use of a Haake Buchler Autodensiflow II (Buchler Instruments, Searle Analytical, Inc., Fort Lee, NJ) gradient maker using KBr salt solutions of d=1.063 and 1.019 at a speed of 1.0 ml/min. Finally, the tubes were topped off with 2.0-3.0 ml of d=1.006 KBr solution containing 1.0 mM EDTA and 1% penicillin/ neomycin/streptomycin. The samples were then centrifuged at 36,000 rpm for 18 hours at 15°C in a Beckman LS Model 350 ultracentrifuge and fractionated into 0.5 ml fractions with the aid of a Gilsen micro-fraction collector. The fractions were then checked for purity by agarose gel electrophoresis and chemical composition analysis; phospholipid, protein, triglyceride, total cholesterol and free cholesterol (1000D microassay, Litton Bionetics, Rockville, MD), with fractions containing the major lipoprotein classes of known density classes being pooled and dialyzed in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA; exclusion limit ca. 3,500) for 3X 24 hours at 4°C in phosphate buffered saline.
Assays of Infectious Virus - the OIF Assay.

Assays for mouse serum or its purified OIF fractions have been previously described (13). Briefly, a dilution of mouse serum or its purified fraction was incubated with approximately 200 focus-forming units (FFUs) of either labeled or unlabeled virus for 1 hour at 25°C and the resulting mixture plated on the appropriate S+L- indicator cells (FG-10). Titers were expressed in 50% inactivating units. The relationship of LDIF concentration was linearly related to virus surviving fraction \((V_n/V_Q)\) (17). Accordingly, OIF units \(O.U. = (0.5) (1 V_n/V_Q) (1/dilution)\). The unit value could be plotted on a log-log paper using \(V_n/V_Q\) values of several serum dilutions. Only values greater than 50.0 O.U./ml are considered specific due to the non-specific lipoprotein/lipid effect previously noted (Nara et al., unpublished observations).

Specific Antisera.

Specific antisera to specific apolipoproteins were graciously obtained from Dr. Aldon J. Lusis, Department of Medicine, UCLA, Los Angeles, CA and included: anti-rat APO E, B and anti-mouse APO A-I. Another antisera tested was rabbit anti-rat APO C-III which was generously supplied by Dr. Walter J. McConathy, Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation, Oklahoma City, OK. One final anti-sera tested was directed against lipoprotein lipase, a major endothelial-bound enzyme critical in the initial stages and completion of lipoprotein metabolism involving serum CHYLOs. This antiserum was obtained from Dr. Michael Schotz, UCLA Medical Center, Los Angeles, CA. All these sera were tested for
their ability to inactivate either OIF or MuLVs when tested in the in vitro OIF-virus neutralization assay.

Preparation of Purified Serum and Thoracic Duct Lipoproteins for SDS-PAGE.

Serum and thoracic duct chylomicrons isolated at \( d<1.006 \text{ g/ml} \) were partially delipidated by rapidly dispersing 1 vol of the chylomicron suspension into 20 vol of ice-cold diethyl ether. After 30 minutes, the mixture was centrifuged at 4°C and the precipitated protein was washed with cold ether. Total delipidation was then accomplished by the addition of 20 vol of ethanol:ether 3:1 (v/v) at 4°C. The next day the mixture was centrifuged and the ethanol:ether was decanted. Residual ether was removed under a stream of nitrogen and the precipitated proteins were dissolved in 0.1M Tris-HCl, pH 8.0, containing 2% sodium dodecyl sulfate (SDS) and electrophoresed in 15% polyacrylamide gels (PAGE). Following electrophoresis, the gels were routinely stained and fixed with Coomassie Brilliant blue G-250 in 45% ethyl alcohol and 10% acetic acid for 10 minutes and afterward destained 2000 ml of a 10% methanol and 0.1% glacial acetic acid mixture.

Because the nature of the factor (i.e., protein, lipid, carbohydrate, etc.) has not been conclusively demonstrated, we evaluated the results of the transfers by a double staining method (14). This technique allows for the Coomassiphilic proteins to initially be evaluated and photographed followed by destaining and restaining with a more sensitive silver nitrate solution (50-100 times more sensitive
than Coomassie blue and approaching the sensitivity of autoradio- 
graphy). This stain also has the advantage of differentially 
staining proteins with charged amino acid side chains and lipopoly-
saccharides (15).

Briefly, gels are photographed after they have been stained by 
Coomassie Brilliant blue G-250 and then placed into 200 ml of a 50% 
reagent grade methanol solution for 2 hours after which the solution 
is changed and the procedure is repeated once. The gels are then 
placed into distilled water for 2 hours with one change of water 
during this time period. The gels are then placed into another 50% 
methanol solution and the previous steps repeated. The gel is 
finally placed into a 30% formaldehyde solution overnight. The gels 
are then stained as described by Wray et al. (14) and photographed 
immediately. The gels could be completely destained by either 
technique at any time with no loss or distortion of the gel proteins.

VLDLs and HDLs were added dropwise to 10 ml of methanol in a 50 
ml conical centrifuge tube. After mixing (by a vortex mixer), 10 ml 
of chloroform was added to the tubes followed by further mixing. The 
tubes were then filled with diethyl-ether and the protein precipi-
tated by centrifugation at 1000 rpm for 5 minutes. Following 
aspiration of the organic solvent, the protein pellet was resuspended 
in 10 ml methanol, the tubes filled with diethyl-ether and recentri-
fuged. This procedure was repeated once; the pellet was washed twice 
with 30-50 ml diethyl-ether and dried under a gentle nitrogen stream. 
Samples were soluble as described above in SDS and loaded into gels.
Iodination of Serum Lipoproteins.

Samples of isolated CHYLOs, VLDLs, and HDLs were radio-iodinated by a variation of the standard iodine monochloride procedure (ICL), at pH 10 as described by Montelaro et al. (16). A stock solution of ICL was prepared by diluting 0.56 g KI, 0.33 g NaIO 3, 29.2 g NaCl, and 21 ml concentrated hydrochloric acid to 250 ml with distilled water. The ICL stock was stable at room temperature for at least one year. Immediately before use, a 1 ml portion of ICL stock was extracted several times for 2 minutes with 3 ml of CCL4 until the CCL4 was no longer colored pint (from I2). Residual CCL4 was removed from the stock by bubbling water-saturated air for 5 minutes. Finally, 1 ml of ICL was mixed thoroughly with 10 ml of 2M NaCl. The LP sample to be iodinated (in 1.0 ml) was diluted with an equal volume of 1M glycine buffer (pH 10) and mixed with 1 mCi of Na 125I (100 mCi/ml, New England Nuclear) in 0.1 ml of glycine buffer. The iodination was initiated immediately with 0.1 ml of ICL and allowed to proceed for 5-10 second. At this time the labeled LP sample was rapidly separated from unreacted 125I by gel filtration through a column (0.9 x 15 cm) of Sephadex G-25 (fine, Pharmacia) eluted with PBS. Using this procedure, at least 70-80% of the incorporated 125I was bound to apolipoproteins, while less than 20-30% was found in the lipid portion. Organic solvent extracts of the various LPs were done in similar fashion.

LP Mixing Experiments.

Active HDLs purified from multiple KBr gradients were used either as un-iodinated or iodinated LPs. HDLs were used for most of
these studies because they could be separated confidently from purified TDCs on these gradients, without the fear of contaminating active LPs. This technique served as the major means for examination of activation of TDCs by serum HDLs. HDLs were concentrated 4X on the KBr gradients, dialyzed extensively in phosphate-buffered saline and used in the mixing experiments. TDCs were purified by three successive KBr gradients and diluted back to original lymph volume prior to use. TDCs and HDLs were mixed in equal volume (2 ml each) and placed on a rotating rack at 37°C in an incubator for 90 minutes. Controls consisted of HDLs and TDCs being mixed separately with equal volumes of sterile saline and treated as above. The same procedure was used for the 125I-labeled HDLs and CHYLOs tested later in these same experiments. Following the incubation period, the various LP mixtures were adjusted to d=1.21 g/ml by the addition of solid KBr in SW-41 centrifuge tubes and prepared for LP separation and isolation as previously mentioned. The samples were centrifuged for 18 hours at 36,000 rpm at 15°C, after which they were fractionated from top to bottom with a Gilson micro-fraction collector. These samples were then rediluted back to original reaction mixture and aliquots were taken for apolipoprotein SDS-PAGE analysis, autoradiography, and virus-inactivating activity (OIF assay).
RESULTS

Thoracic Duct Purification.

TDC isolated by microannulation were purified by KBr gradient density ultracentrifugation and examined by agarose gel electrophoresis and SDS-PAGE. The characterization of TDL lipoproteins isolated from one KBr gradient (1/2 ml fractions) was accomplished by agarose gel electrophoresis and are presented in Fig. 5.1. Lane 1 represents the top of the gradient (d<1.006 g/ml) and consisted of a white creamy/waxy material which stained red for cholesterol, lipid and triglyceride using an Oil Red O stain. The material in and around the well are large TDC while the material present as a smear beyond the well are interpreted as smaller chylomicrons or very low density lipoproteins (beta to pre-alpha region). Unfortunately, no published information on mouse TDL or TDC could be found for comparison. Careful examination of the gel in lanes 10, 11 and 12 reveal a faintly staining band at the extreme edge of the gel (alpha region) and probably represents recirculating HDLs. The material seen in lane 25 is due to residual floating material (lane 1 and 2) which was not completely collected. Only slight amounts of OIF activity (50-100 O.U./ml) were found in fractions 10, 11 and 12 (data not shown).

The SDS-PAGE apolipoprotein profile for the same fractionated TDL is presented in Fig. 5.2. Again, the top of the gradient (d<1.006 g/ml) is represented by lane 1 and consists primarily of large chylomicrons and VIDLs. The two major apolipoproteins present are APO A-IV at 48 kilodaltons (kD) and APO A-I (28 kD). The protein
found at 69 kD was identified by radioimmunoprecipitation to be albumin. The band at the top (>200,000) probably corresponds to APO B, while the apolipoproteins/proteins less than 14 kD remain unidentified. These may correspond to some of the APO C's reported to be present in TDCs. The finding of larger amounts of APO A-IV is typical of rat TDCs and newly secreted serum chylomicrons of many other species (17,18). APO A-I is the major apolipoprotein species generally found associated with circulating HDLs and has been reported to be the major protein associated with TDC (18).

TDCs were purified from TDL (collected by microannulation) by multiple KBr gradient centrifugations and characterized by SDS-PAGE for apolipoprotein content (Fig. 5.3). Lane 1 is representative of undiluted TDL and serves to show the tremendous amount of protein present in lymph. Lanes 2 and 3 are apolipoproteins associated with TDCs isolated after one and two KBr gradients, respectively. TDCs were found to undergo protein shedding between successive gradients and has been reported to occur by others (17). This characteristic allowed for purification of OIF-negative precursors (TDCs) to be used in the transfer experiments.

**Serum Lipoprotein Purification.**

Serum lipoproteins isolated from both fasted and oil gavaged BALB/c were characterized by both agarose gel electrophoresis and SDS-PAGE analysis and are presented in Figs. 5.4 and 5.5, respectively. Fasted sera (Fig. 5.4A) contains predominantly VLDLs (smeared positive lipid staining region in lanes 2, 3 and 4) and some CHYLO remnants (small staining line found in the well). Intermediate and
low density lipoproteins (LDLs) (pre-beta and beta region) are found in lanes 5, 6, 7, 8 and 9. The HDLs which make up the bulk of fasted serum lipoproteins (approximately 77%, unpublished data) are found beginning in lane 10 and extending to lane 19 (d=1.063-1.021 g/ml), (lanes 1 and 18 are albumin markers) and are characterized by their intensely staining alpha migrating band. Lipoproteins isolated from oil gavage mice exhibit a more pronounced band of lipid staining material remaining in the well (lane 2, Fig. 5.4B) and represent newly secreted large chylomicrons. Lanes 3-10 are characterized by elongated beta and pre-alpha staining regions and represent a heterogeneous collection of metabolized CHYLOs, VLDLs and LDLs following oil-induced lipemia. The HDLs are present again starting at lane 11 and continued to lane 21 (d>=1.21 g/ml).

Apolipoprotein profiles for fasted and oil gavaged mice are presented in Fig. 5.5A and B, respectively. Similar protein profiles are seen for both sera with APO Bs (>200 kD, lanes 1a and b only), APO A-IV (46 kD, lanes 1a and b only), APO E (35 kD, lanes 1a and b, 2a and 2b, and 3b only), APO A-I (28 kD, lanes 5a-15a and 7b-14b) and APO Cs (<14 kD, lanes 1a and b, 5a-15a and 7b-14b).

Effect of Various Anti-Apolipoprotein Antisera on OIF and Virus.

Only a limited number of investigators have isolated, purified and raised antibodies to any murine apolipoproteins. All antisera were uniformly negative for their ability to exhibit any anti-OIF effect. This list included: mouse APO A-I, the major protein of TDCs and HDLs; rat APO E (35 kD protein associated with serum CHYLOs), rat APO B (>200 kD protein of serum CHYLOs), and rat APO C-III (9.6 kD
protein associated with serum CHYLOs, VLDLs and HDLs). All these sera have been shown to cross-react by double immunodiffusion, immunoelectrophoresis and radioimmunoprecipitation techniques (19). Also tested were the effect of anti-bovine and rat lipoprotein lipase on E- and RM-MuLVs. Lipoprotein lipase is the major endothelial-bound enzyme responsible for catabolism of serum CHYLOs and was considered as a possible mechanism of OIF. No effect was noted when these antisera were incubated with either virus or OIF-associated lipoproteins (data not shown).

**Effect of Iodination on Purified OIF Lipoproteins.**

Purified TDCs and serum OIF LPs were iodinated and tested for their biological activity and apolipoprotein profiles. Iodination had no effect on LP migration in agarose gels (Fig. 5.6). Lanes 2, 3 and 4 were taken from the same material that was used in Fig. 5.4, lanes 2-4. Also, iodination had no effect on OIF-associated LP's ability to specifically inactivate RM-MuLV (data not shown). When initially tested, however, iodinated LPs used in the OIF assay were allowed to remain in the tissue culture dishes. This resulted in extensive cellular alterations of the monolayers due to gamma radiation, this precluded the evaluation of iodinated OIF LPs to inactivate virus. To circumvent this problem the iodinated OIF/virus incubations were washed from the cell monolayers after 60 minutes (a time period previously determined to be adequate for the production and/or inhibition of plaques). Radio-iodinated OIF did not effect E-MuLV in these assays and thus rules out radiation as a mechanism of viral inactivation.
SDS-PAGE of the iodinated TDCs (1 KBr), CHYLOs and HDLs (2 KBr) are presented in Fig. 5.7. Lanes 2 and 3 are CHYLOs and HDLs, respectively, and reveal the typical apolipoprotein profile for their respective species. Note the prominent radioiodinated protein(s) (<14 kD) found in both OIF containing LPs and the absence of this corresponding band in the TDCs.

**Effect of LP Mixing Experiments on the Transfer of OIF to TDC.**

The biological activity profiles for LP mixing experiments with various OIF positive and negative LPs are presented in Table 5.1. TDCs mixed with PBS display no virus-inactivating activity. The concentrated HDLs, on the other hand, isolated from OIF-positive BALB/c serum exhibit generally 1,000 to 3,000 O.U./ml in fasted serum. An unexpected finding was the enhanced OIF activity seen following the incubation of TDCs and HDLs. The HDLs used in the transfer contained approximately 2,000 O.U./ml, however, following the incubation with TDCs and their re-isolation, an almost 3-fold increase in TDC associated OIF activity (5,810 O.U./ml) occurred. Also as surprising, was the inapparent loss of total activity of OIF in the HDL fraction re-isolated following the incubation. This may suggest an excess of factor associated with HDLs and a saturable (limited) ability of HDLs to inactivate virus. Other fractions (d>1.21 g/ml) were unable to transfer any activity to TDCs during an identical incubation.

Due to the enhancing effect noted by TDCs with the homologous HDLs, it was decided to test it against other known OIF-negative exotic mouse strains previously reported (Nara et al., unpublished
observations). To test this by LP mixing experiments allowed for the detection of possible OIF associated HDLs that might be in an inactive state capable of "OIF activation" following the transfer to BALB/c TDCs. As can be seen in Table 5.1, no OIF activity resulted from the incubation of either HDLs or serum fractions (d>1.21 g/ml) and confirms that exotic mouse strains are truly OIF negative.

**Transfer of OIF Activity and Protein(s) to TDCs Following LP Mixing Experiments.**

Aliquots taken from those previously used in the OIF determinations following the LP mixing experiments were examined by double staining SDS-PAGE analysis and are presented in Fig. 5.8. The gels were initially stained by Coomassie blue (lanes 6-9), photographed, and evaluated for protein(s) transfer to the TDCs. Lane 6 represents the 2 KBr purified TDCs which demonstrate the two major Coomassiephilic proteins (APO A-IV at 48 kD and APO A-I at 28 kD) previously mentioned. Lanes 8 and 9 are similarly purified HDLs and CHYLOs, respectively, which also exhibit their previously mentioned apolipoprotein profiles. Attention was paid to the low molecular weight protein(s) (<14 kD) as all proteins larger were effectively ruled out in these and earlier studies (Nara et al., unpublished observations). The low molecular weight Coomassiephilic protein(s) from CHYLOs were found as a diffuse blue band usually extending from 14 kD to less than 6 kD. The HDLs (lane 8) also had a lightly Coomassiephilic band starting at 14 kD but developed a more discrete band with greater intensity around 4 to 6 kD. This band could be demonstrated to transfer to the TDCs following the incubation (lane 7) and was
completely missing from native TDCs (lane 6). Also transferred, based on relative amounts of protein (densimetric scanning), was APO A-I.

The distribution of argyrophilic apolipoproteins are presented also in Fig. 5.8, lanes 2-5. Coomassiphilic proteins from TDCs, as described previously, appear more contrasted and a number of "other" uncharacterized proteins can be detected between 48 and 200 kD. No proteins were detected in regions less than 21 kD even after extreme over-developing of the silver staining reaction (gel not shown). The same overall differences were noted for apolipoproteins from HDLs and CHYLOs (lanes 4 and 5, respectively). A number of protein bands were more apparent following the silver stain, however, only those less than 14 kD were considered significant based on earlier rule-outs. For example, the argyrophilic protein band just under the large 28 kD (APO A-I) protein band (Fig. 5.8, lane 4) is present also in isolated CHYLOs (lane 5) and VLDLs (not shown). This band was demonstrated in earlier experiments (data not shown) to be reduced in significant amounts following purification by multiple KBr gradient centrifugations and not correlate to biologic activity. The other large argyrophilic band (21 kD) found in lane 5 and associated with CHYLOs is not present in OIF-positive HDLs (lane 4) and, therefore, effectively rules this protein out. The major argyrophilic protein(s) transfer(s) to occur between the HDLs (lane 3) and the TDCs (lane 3), involve a prominent band which starts just under the 14 kD molecular weight marker and extends to approximately 8 kD. A minor protein transfer also occurred just above this protein in the range of 16 kD.
Again, no other bands were detected to transfer following over staining of the gel. Also, fractions taken from the top of the control HDL gradient (same as those collected for the TDCs) revealed no protein bands at any position in the gel and serve to verify that the protein transfers were specific and mediated by the HDLs.

To test for other non-Coomassiphilic and non-agryrophilic proteins that may be involved in these transfers, $^{125}$I HDLs were evaluated for their ability to transfer radiolabeled proteins and OIF activity. Normal $^{125}$I-labeled HDLs characterized by agarose gel electrophoresis (Fig. 5.6, lanes 10-13) are presented in Fig. 5.9, lane 2. Again the major apolipoproteins (APO A-I, APO A-II, APO C's) appear in their appropriate positions in the gel. Lane 4, represents the material taken from the top of the gradient containing iodinated HDLs. This served as a control for the spontaneous formation of iodinated LPs which might have undergone a spontaneous alteration in their densities and be associated nonspecifically with the fractions collected at $d<1.006$ g/ml. A small amount of APO A-I (28 kD) protein was detectable after prolonged exposure times (2X the time used in lanes 1-3). It appears that the TDCs again were transferred a protein of approximately 8 to 14 kD with a minor band occurring at approximately 6 kD. No differences were seen in the HDL protein profiles when isolated from the TDC gradient (data not shown). Also, because no 6 kD protein was detected in the $^{125}$I-labeled HDLs (lane 2), the band detected after incubation with TDCs may be the result of enzymatic degradation (i.e., proteases) or other
as yet unexplained occurrences. This 6 kD band may be similar to that detected by Coomassie staining in the previous experiments.
OIF has been described by Levy et al. (7) to transfer from mouse LPs to human LPs and further suggested by Kane et al. to be associated with the C apolipoproteins (6). No physical evidence, however, for these protein transfers has ever been published and, as such, has been purely by tracking biologic activity. In this study we have demonstrated that, in fact, protein(s) due transfer to endogenously produced TDCs and, as such, convey biologic activity (specific virus inactivation - "OIF"). The use of mouse TDCs allows for a homologous natural OIF-negative LP precursor to interact with OIF-positive serum LPs and confer virus-inactivating activity as it might in vivo. The activation of TDCs, however, is very inefficient for the following reasons. First, as reported earlier (Nara et al., unpublished observations) an equivalent volume and concentration of freshly isolated serum CHYLOs when compared to the same amount of TDC, would have 12,000-fold the activity. In the experiments presented here, HDLs were used at 4X the normal serum concentration and TDCs used at original lymph volume. This only resulted in an increase in OIF levels approaching 100-fold. The ratio of the reaction used in this study was in excess of that which occurs in the body to yield such elevated OIF levels. More specifically, the average circulating blood volume of the laboratory mouse is approximately 5.77 ± 1.0 ml/100 g body weight depending on the strain (20). The average production of TDL in our hands and reported by other (11; Nara et al., unpublished observations) is approximately 1 ml/hour. This
calculates out to approximately 3 volume changes per 24 hours in the mouse. Also, during any one hour theoretically 1 ml of lymph will mix with approximately 6 ml of blood. Cholesterol levels are directly related to HDL concentration in the mouse (21,22) and, as such, can be used in the calculation of the lymph/serum ratio. A milliter of fasted serum contains approximately 1.0 mg of cholesterol with approximately 6.0 mg of total circulating cholesterol (HDLs contain about 85% of the total serum cholesterol at any one time) available for transfer. The concentration of our HDLs 4.8 mg/ml with two milliters being used in the reaction. Thus, it appears that the magnitude of activation seen associated with serum CHYLOs following oil gavage is due to some other additive or synergistic mechanism.

The apolipoproteins of interest here are known to associate with newly secreted CHYLOs by both direct transfer from HDLs and their association with the liver and splenic sinusoids and/or other microcapillary beds (i.e., adipose, pulmonary tissue) before they can be metabolized (17,18). This tissue/endothelial interaction was alluded to in our earlier report (Nara et al., unpublished observations) and appears to be necessary for the maximal OIF activation of newly secreted CHYLOs. Of the potential apolipoproteins which might be responsible (APO C-I, II, III, and APO A-II), APO C-III (mouse type is estimated to be about 9.6 kD) can be partially ruled out based on the results of the rabbit anti-rat APO C-III experiments. The characterization of the remaining mouse apolipoproteins with the exception of APO A-II is still needed. Mouse APO A-II has been shown to be approximately 11-12 kD and its function in any species is still
unknown. APO C-I and II found to transfer to CHYLOs have been shown in many species to be predominantly associated with the HDLs (8). Our studies indicate that transfer from HDLs only does not approach the efficacy of the reaction experienced in vivo. Also reported earlier, was the finding that if fresh blood (up to a ratio of TDC to blood of 1:8) were used in the incubation of TDCs (a condition most resembling the body), no enhancement of OIF activation occurred (Nara et al., unpublished observations). Perfusion studies are under way to answer this question. In conclusion, our studies indicate that OIF activity is associated with a major transfer of an argyrophilic and radio-iodinatable 8-12 kD protein and a minor 6 kD, Coomassiphilic protein to TDCs. Activation of OIF, however, may require the transfer of both proteins. Now that a class of protein(s) has been identified to transfer and are consistently associated with virus inactivating activity, isolation and purification schemes are currently being investigated to determine more specifically the identity of this protein.

Another interesting correlation may exist between murine retroviruses and lipoprotein metabolism in this species. The structural gene for mouse APO A-II, designated Alp-2, resides on mouse chromosome 1, tightly linked with Ly-m20, a lymphocyte alloantigen locus (23). This same locus has been shown to map to several xenotropic proviral genes (Bxv-1, Mtv-7, Mtv-10 and Sxv), the last of which has been shown to be a dominant gene which may control susceptibility of wild mouse cells by encoding a wild mouse variant of the MCF receptor which allows penetration by xenotropic MuLVs (24,25). This suggests
a prior genetic recombinational event may have occurred between mouse lipoprotein metabolism genes (i.e., APO A-II) and endogenous MuLVs such that the viral receptor is synthesized as a part of HDLs apolipoprotein and/or also synthesized acquired through endothelial association by newly secreted CHYLOs. This biochemical symbiosis may be required by the species for normal lipoprotein metabolism or as a coincidental occurrence, thereby indirectly resulting in the amplification of the X-MuLV receptor on virtually all the circulating lipoproteins present in the body. It would thus become apparent why no endogenous X-MuLV can be isolated due to the constant inactivation by X-MuLV bearing LPs. A further piece of evidence was reported earlier and suggests the possible correlation between endogenous MuLV-related sequences and the presence of OIF in various exotic mouse strains (Nara et al., unpublished observations). Genetic studies involving this locus are under way to determine the relationship of endogenous X-MuLV sequences and the presence of OIF.
SUMMARY

Lipoprotein associated oncornavirus inactivating factor (OIF) is associated with serum-derived chylomicrons (CHYLOs), very low density lipoproteins (VLDLs), and high density lipoproteins (HDLs) and absent from CHYLOs isolated from the thoracic duct (TDCs). Partial activation appears to be related with the acquisition of low molecular weight protein(s) derived from circulating HDLs. No transfer occurs with serum fractions greater than a density of 1.21 g/ml. An 8 to 12 kD argyrophilic protein(s) and a 6 kD Coomassiphilic protein band were found to transfer to purified TDCs from concentrated purified serum HDLs. Only the 8 to 12 kD protein band was found to transfer when iodinated HDLs were used in similar experiments. A 100-fold increase in OIF activity was detected following the incubation and re-isolation of TDCs. TDCs were determined to contain none of the transferable low molecular weight (<14 kD) apolipoprotein following multiple gradient purification, and suggest that this/these protein(s) are required for the retroviral inactivating properties of OIF. HDLs purified from exotic OIF-negative mice do not transfer virus-inactivating activity to TDCs.
Table 5.1 Transfer of OIF by Various Purified Murine Serum Lipoprotein Fractions to TDC

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>d&lt;1.006 (CHYLO, VLDLs)</th>
<th>d=1.006-1.063 (IDL, LDLs)</th>
<th>d=1.063-1.210 (HDLs)</th>
<th>d&gt;1.210 (serum protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDCs + PBS</td>
<td>45 ± 20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52 ± 10</td>
<td>47 ± 12</td>
<td>51 ± 8</td>
</tr>
<tr>
<td>HDLs + PBS</td>
<td>49 ± 20</td>
<td>115 ± 52</td>
<td>2,125 ± 350</td>
<td>72 ± 10</td>
</tr>
<tr>
<td>TDCs + HDLs (active)</td>
<td>5,810 ± 1,200</td>
<td>253 ± 75</td>
<td>2,124 ± 400</td>
<td>130 ± 30</td>
</tr>
<tr>
<td>TDCs + Fxs (&gt;1.210 g/ml)</td>
<td>61 ± 15</td>
<td>58 ± 9</td>
<td>61 ± 13</td>
<td>49 ± 21</td>
</tr>
<tr>
<td>Fxs (&gt;1.210 g/ml) only</td>
<td>59 ± 5</td>
<td>42 ± 10</td>
<td>39 ± 5</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>TDCs + HDLs (non-active)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41 ± 8</td>
<td>39 ± 5</td>
<td>37 ± 10</td>
<td>29 ± 10</td>
</tr>
<tr>
<td>TDCs + Fxs (&gt;1.210 g/ml)</td>
<td>37 ± 2</td>
<td>39 ± 4</td>
<td>50 ± 7</td>
<td>42 ± 7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Value is expressed in 50% inactivating units. The relationship of OIF concentration is linearly related to the virus surviving fraction (V<sub>n</sub>/V<sub>0</sub>). Accordingly, O.U./ml = 0.5 (V<sub>n</sub>/V<sub>0</sub>) (1/dilution). The unit value can also be graphically plotted on a log-log paper using V<sub>n</sub>/V<sub>0</sub> values at several serum dilutions.

<sup>b</sup> HDLs were isolated from a previously characterized OIF (-) exotic mouse (Mus pahari).
Fig. 5.1 Distribution of thoracic duct lipoproteins isolated by potassium bromide gradient density ultracentrifugation and examined by 0.5% agarose gel electrophoresis. The lanes are in numerical order and represent lipoproteins isolated sequentially from their lightest density, represented by lane 1 ($d_{<1.006\text{ g/ml}}$) to their greatest density, lane 25 ($d_{>1.21\text{ g/ml}}$). The gel was stained with Oil Red O for the detection of cholesterol and lipid.
Fig. 5.1
Fig. 5.2 Distribution of apolipoproteins associated with thoracic duct lipoproteins isolated by potassium bromide gradient ultracentrifugation, delipidated and analyzed by 13% sodium-dodecyl polyacrylamide gel electrophoresis and stained with Coomassie blue. The lanes are in numerical order and represent lipoproteins isolated from their lightest density, represented by lane 1 (d≤1.006 g/ml) to their greatest density, lane 17 (d≥1.21 g/ml).
Fig. 5.2

KBr Gradient of TDL
Fig. 5.3 A 13% SDS-polyacrylamide gel electrophoresis analysis of thoracic duct chylomicrons (TDCs) isolated from native thoracic duct lymph by successive potassium bromide (KBr) gradients. Lane 1 contains whole thoracic duct lymph. Lane 2, represents the apolipoproteins present in purified TDCs after one KBr gradient. Lane 3, represented the TDC apolipoproteins remaining after a total of three successive KBr gradients.
Fig. 5.4 Distribution of serum lipoproteins (LPs) isolated from adult BALB/c mice by potassium bromide gradient ultracentrifugation and analyzed by 0.5% agarose gel electrophoresis and stained with Oil Red O. Gel A contains LPs isolated from 18 hour fasted serum. Gel B contains serum from animals following oil gavage. The lanes are in numerical order and represent LPs isolated from their lightest densities, lane 1 (d_1.006 g/ml) to those at the bottom of the gradient (d>1.21 g/ml).
Fig. 5.5 Distribution of apolipoproteins from serum lipoproteins (LPs) isolated from adult BALB/c serum by potassium bromide gradient ultracentrifugation and analyzed by 13% SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Gel A contains apolipoproteins from delipidated LPs isolated from 18 hour fasted sera. Gel B contains similarly prepared apolipoproteins from serum obtained following oil gavage. The lanes are in numerical order and represent LPs isolated from their lightest densities, lane 1 (d<1.006 g/ml) to those at the bottom of the gradient, lane 18 (d>1.21 g/ml).
Fig. 5.5
Fig. 5.6 Autoradiograph of an 0.5% agarose gel containing $^{125}$I-labeled serum lipoproteins (LPs) from fasted BALB/c mice. These were previously isolated by potassium bromide gradient ultracentrifugation and radioiodinated as described in Materials and Methods. Lanes are in numerical order and represent the following LPs: Chylomicrons (origin) and very low density LPs (pre-alpha), lanes 1-3; intermediate density and low density LPs, lanes 5-9; and high density LPs (alpha), lanes 10-14.
Fig. 5.7 Autoradiograph of the apolipoproteins from various $^{125}$I-labeled, delipidated lipoproteins isolated from BALB/c mouse thoracic duct lymph and serum by potassium bromide (KBr) gradient ultracentrifugation. Lane 1 represents the apolipoproteins from chylomicrons isolated from 1 KBr gradient; lane 2 contains apolipoproteins from serum chylomicrons also isolated from 1 KBr gradient; lane 3 contains apolipoproteins from high density lipoproteins isolated from the same gradient as the previous lane.
Intestinal Lymph ($^{125}$I)
Serum Chylo's ($^{125}$I)
Serum HDL's ($^{125}$I)
Fig. 5.8 SDS-polyacrylamide gel of OIF transfer experiments utilizing a double-staining technique for the evaluation of apolipoprotein transfer(s) to thoracic duct chylomicrons (TDCs). Gel A (silver stained) contains the following: lane 1, molecular weight markers; lane 2, TDCs only; lane 3, TDCs reisolated following an incubation with high density lipoproteins; lane 4, high density lipoproteins only; lane 5, chylomicrons only. Gel B is the same gel stained with Coomassie blue.
Fig. 5.8
Fig. 5.9 SDS-polyacrylamide gel autoradiograph of transferred $^{125}\text{I}$-labeled high density lipoprotein (HDLs)/apolipoprotein to thoracic duct chylomicrons (TDCs). Lane 1 represents fractions collected from the top of the control TDCs; lane 2 represents normal $^{125}\text{I}$-labeled HDLs; lane 3 represents TDCs reisolated from a potassium bromide gradient following the incubation of iodinated HDLs; lane 4 represents material taken from the fractions ($d<1.006$) of control iodinated HDLs from the gradient described for lane 1 and exposed to film twice as long as those required for lanes 1-3.
REFERENCES


BIBLIOGRAPHY


Mahley, R.W., Innerarity, T.L., Rall, S.C., and Weisgraber, K.H.
Mathews, T.H.J., Lawrence, M.K., Nair, C.D.G., and Tyrell, D.A.J.


