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AUTOIMMUNITY AND OTHER IMMUNE MECHANISMS IN
RABBITS WITH EXPERIMENTAL TRYPANOSOMA CONGOLENSIS
INFECTIONS.

The Ohio State University, Ph.D., 1971
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AUTOIMMUNITY AND OTHER IMMUNE MECHANISMS IN RABBITS
WITH EXPERIMENTAL TRYPANOSOMA CONGOLENSIS INFECTIONS

DISSERTATION

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

By

John Michael Mansfield, B.A., M.A.

* * * *

The Ohio State University

1971

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Department of Microbiology
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INTRODUCTION

Trypanosomiasis has been a serious barrier to the economic development of tropical and subtropical Africa. Pathogenic trypanosomes have destroyed the lives of man and his domestic animals and have thwarted the colonization of vast areas of land that could otherwise be fertile farmland and pasturage.

In regions of Africa where the trypanosome is endemic, overt disease flares up only when man and his animals move into areas where the vectors and wild animals that transmit or harbor the parasites live. In addition, drug-resistant strains of trypanosomes are constantly arising in areas where chemotherapy programs have been used to keep outbreaks of trypanosomiasis under control. Goodwin (1970) compares the dread disease to a dragon that "...sometimes slumbers and smoulders quietly for long periods but at any moment it may awaken and cause widespread loss of life to man and his animals."
Nagana, or tsetse fly-transmitted trypanosomiasis of domestic animals, was first recorded in the early days of European colonization of Africa. Boer settlers described ravages of their livestock by the tsetse fly in the 1830's (U.S. Livestock Sanitary Association, 1954). Livingstone (1860) had many encounters with the tsetse fly during his travels in the Zambesi basin of Africa, and remarked that "...it is well known that the bite of this poisonous insect is certain death to the ox, horse and dog."

Nagana is caused by infections with Trypanosoma congolense, T. dimorphon, T. simiae, T. suis, T. vivax, T. uniforme and T. brucei. The course and severity of the disease vary with the trypanosome species as well as with the species of animal that is infected. T. congolense and T. vivax are considered to be the species most pathogenic for cattle (Fiennes, 1950; U.S. Livestock Sanitary Association, 1954; Davey, 1958; Richardson and Kendall, 1963; Gell and Coombs, 1968; Killick-Kendrick, 1971).

Trypanosoma congolense may be differentiated from the other trypanosome species that infect cattle on the basis of morphology, mode of transmission, mode of reproduction in the tsetse fly (Glossina spp.) and the

The typical trypomastigote morphology of *T. congolense* in the blood of a mammalian host is visible in Figures 1 and 2. The posterior end of the parasite is rounded. A flagellum arises from an internal basal body near the kinetoplast in the posterior end, and emerges as an undulating membrane-bound organelle running the length of the parasite to the anterior end; typically there is no free flagellum extending from the anterior end. Total body length ranges from 9 to 18 µm, with an average length of about 14 µm, in the mammalian host. Body width is approximately 1 to 2 µm.

In nature, *T. congolense* is cyclically transmitted by several species of Glossina (mainly *G. morsitans*, *G. palpalis*, *G. longipalpis* and *G. tachinoides*) from infected animals to other domestic and wild animals. After a tsetse fly feeds on the blood of an infected animal, the ingested trypanosomes reproduce by longitudinal binary fission and undergo morphologic variation in the fly's midgut. Elongate trypomastigote forms are evident in the
FIGURE 1

*Trypanosoma congolense* in the blood of rat; the typical trypomastigote morphology is represented.

(X 1000)
FIGURE 2

Carbon replicas of *Trypanosoma congolense* taken from infected rat blood. P = posterior end of the parasite; A = anterior end; F = flagellum; UM = undulating membrane, encompassing the flagellum. (Photograph courtesy of Thomas M. Seed)
midgut as well as epimastigote forms (Figure 3). Epimasti-
gote forms may be found in the proboscis to which they
migrated from the midgut; the epimastigotes in the pro-
boscis transform into metacyclic forms which are infective
for mammalian hosts. The time required for the production
of metacyclic forms from the ingested blood forms is about
three weeks. Once infected, a fly remains infected for
life. The presence of the parasites normally does no
harm to the fly. At the next blood meal, infective
metacyclic forms are discharged with the saliva into a
new mammalian host. The metacyclic forms transform into
trypomastigote forms and commence dividing by longitudinal
binary fission in the blood. When mechanical syringe
passage of a trypanosome strain through susceptible labora-
tory animals is carried out, conversions to epimastigote or
metacyclic forms do not occur.

Trypanosoma congolense is infective in small numbers
when ejected from Glossina spp. into experimental animals,
but no accurate ID₅₀ has been established for cattle
(Wilson et al., 1966). The geographic incidence of T.
congolense infections corresponds, predictably, to the
geographic range of Glossina spp. The area concerned
FIGURE 3

Morphological variations of *Trypanosoma congolense* as seen in *Glossina* spp. (From Baker, 1969)

Trypomastigote Form  Epimastigote Form  Metacyclic Form
includes West, Central and East Africa, and lies between 14° N and 29° S latitudes -- a larger area than the continental United States (Lumsden and Wells, 1968).

The incidence and severity of *T. congolense* infections are compounded by the existence of reservoir host populations of wild animals. In animals like the wild antelope, giraffe, eland and wildebeast, *T. congolense* may be found coexisting with the host in a nonpathogenic state. In addition, drug-resistant strains of *T. congolense* occur in areas where chemotherapy has been instituted, and some of these strains are cross-resistant to therapeutic doses of trypanocidal drugs to which they have not been exposed (Jones-Davies and Folkers, 1966; Mwambu, 1966; Leach, 1969). Recent evidence shows that drug resistance in *T. congolense*, once attained, is a stable characteristic which does not alter infectivity for *Glossina* spp., nor transmissability (Gray and Roberts, 1971).

Domestic cattle infected with *T. congolense* are unable to rid themselves of the parasite. The trypanosomes in the blood of an infected animal alter their surface antigens so that the end result is the appearance of a succession of antigenically distinct trypanosome populations (Wilson, 1966; Wilson and Cunningham, 1970). At any time during
infection, therefore, specific antibody for all antigenic variants which occurred preceding serum sampling may be found, but no antibody is found for antigenic variants arising later in the infection. Common antigens have been noted, however, among the variant populations in experimental infections (Wilson and Cunningham, 1970; Mahmoud and Kreier, 1971). Overall, the situation is analogous to the well-documented antigenic polymorphism exhibited by *T. brucei* (Desowitz, 1961; Williamson and Brown, 1964; Gray, 1965).

The biologic activity of antibodies produced to trypanosomes is of the same nature as antibodies produced to bacterial pathogens, for example. Agglutinins, lysins, opsonins and immobilizing (anti-flagellar) antibodies have been found in the sera of trypanosome-infected animals (Weinstein, 1967; Taliaferro and Stauber, 1969; Desowitz, 1970; Ogilvie, 1970). Antibodies to trypanosome antigens are primarily of the IgM and IgG classes; IgM levels are abnormally high in trypanosome-infected man and animal and these elevated levels may be due, in part, to the succession of antigenic variants against which the animals must produce antibody (Mattern, 1964;
Cunningham et al., 1966; Smithers, 1967; Rees, 1969; Ogilvie, 1970). The antigenic diversity of trypanosome populations and, hence, the diversity of antibody specificities produced to them has hindered attempts at vaccination and serodiagnosis. The latex fixation test of Mahmoud and Kreier (1971), which uses non-variant internal antigens, may provide one answer to the problem.

Trypanosoma congolense is readily infective for laboratory animals, unlike T. vivax with which it is very difficult to produce experimental infections and which is inconsistently pathogenic. The symptoms and pathology of T. congolense infections vary with the infecting strain and the species of animal that is infected. Small rodents such as rat or mouse usually show no external signs of disease when experimentally infected, and death occurs within several days or may be delayed somewhat depending upon the infecting strain (Binns, 1938; Fiennes, 1950; Irfan, 1968). Death is preceded by massive parasitemia; there may be as many trypanosomes as erythrocytes present in the blood. In these acute infections trypanosomes are found only in the blood (Losos and Ikede, 1970), and death probably results
from mechanical blockage of vessels and a systemic metabolic imbalance since parasite oxygen uptake and glucose degradation are quite high (Agosin and Von Brand, 1954; Von Brand and Tobie, 1959; Fulton, 1969).

Rabbits with experimental *T. congolense* infections may undergo a chronic, necrotic disease with few trypanosomes present extravascularly (Losos and Ikede, 1970; Wosu, 1971). Death usually occurs after several weeks of infection.

In a clinicopathologic study of *T. congolense*-infected rabbits, Wosu (1971) found external lesions around the ears, eyes, nose and genitalia, as well as weight loss and lowered hematocrit values. Symptoms of disease occurred as rising titers of antibody to parasite antigens appeared. Although serum glucose levels decreased during infection, blood urea nitrogen, serum sodium and potassium, creatine and total serum protein levels were normal in most rabbits. Macroscopic lesions were not consistently found on internal organs, but microscopic inflammatory changes were observed in kidney and lung. Sections of necrotic skin and genital tissue revealed edema, perivascular accumulation and foci of
neutrophils and mononuclear cells in the tissues and extensive vascular disruption.

Naturally infected cattle may have an acute or chronic disease that ends in death (Fiennes, 1950). Symptoms are relapsing fevers, reduced hematocrit values, emaciation and coat roughness, prostration and weakness, vomiting and diarrhea. Large numbers of trypanosomes periodically appear in peripheral blood, and these appearances are followed by sudden decreases in parasite numbers and an increase in monocytic cells in the blood. Death occurs most frequently when body temperature falls, when parasites are few in the blood and antibody is demonstrable to them, and when monocytes appear in large numbers in the blood.

Histological analyses of tissues from stricken cattle reveal extensive tissue disruption and necrosis (Fiennes et al., 1946; Fiennes, 1950). Kidney, liver, pancreas, pituitary and lung necrosis is widespread, and mononuclear cell infiltrates are observed in several tissues; vascular occlusion occurs frequently. Trypanosomes are found primarily within the blood vessels, although Fiennes (1950) found the parasites in lymph nodes, adrenal
The mechanism(s) responsible for pathology and death in *T. congolense* infections have not yet been elucidated. Lavier (1940) attributed blockage of heart vessels of infected cows to agglutinated trypanosomes, and suggested that this may be a contributory cause of death. Fiennes (1950) suggested that direct parasite action, intoxication or "...excessive bodily reactions to the parasite or its breakdown products" may occur. More recently, Fulton (1969) states that "The mechanism by which trypanosomes damage their hosts is still far from clear in spite of the considerable amount of work devoted to this problem, mostly in infections with pathogenic African trypanosomes. Three opposing views have been put forward: (1) that a toxin was responsible, although this has never been proved; (2) that exhaustion of blood sugar and glycogen reserves occurred, also unproved; (3) that internal asphyxia occurred."

Several observations may be drawn from a literature survey on African trypanosomiasis:

(a) Experimental evidence, or the lack of it, tends to rule out metabolic upsets, direct action of
the parasites or trypanosomal toxin as major causes of tissue damage and death in animals with chronic *T. congoense* infections.

(b) There are no demonstrable intracellular stages of *T. congoense* that could contribute to tissue disruption in mammals with chronic infections.

(c) The trypanosomes are present in low numbers, or are undetectable, in the blood of chronically infected animals when the greatest observable tissue damage or death occurs.

(d) Symptoms and pathology of disease could be related to immunopathologic mechanisms as evidenced by neutrophil and mononuclear cell infiltrates in various tissues, kidney and lung damage, vascular lesions and a correlation between rising anti-parasite antibody and tissue damage.

(e) Although much work has been done on the immunopathology of infectious diseases, the author is unaware of any published reports on potentially pathologic immune mechanisms occurring in natural or experimental *T. congoense* infections.
Preliminary investigations of such mechanisms have been carried out on animals with infections of the Brucei group trypanosomes; however, the Brucei group trypanosomes differ substantially in a number of ways from the Congolense trypanosomes and are relatively non-pathogenic for cattle (Figure 4).

The preceding observations merited a search for potentially immunopathologic mechanisms occurring in chronic *T. congoense* infections. Therefore, the experiments presented in this dissertation were designed to detect the presence or absence of humoral and cell-mediated autoimmunity to normal tissue antigens, and to test for immediate or delayed hypersensitivity to trypanosome antigens in rabbits with *T. congoense* infections.
FIGURE 4
Comparisons of Brucei group and Congolense group trypanosomes

<table>
<thead>
<tr>
<th>Subgenus</th>
<th>Brucei Group</th>
<th>Congolense Group</th>
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<tr>
<td>Spp. transmitted by Glossina</td>
<td>Trypanozoon</td>
<td>Nannomonas</td>
</tr>
<tr>
<td></td>
<td>T. brucei, T. gambiense, T. rhodesiense</td>
<td>T. congolense, T. dimorphon, T. simiae</td>
</tr>
<tr>
<td>Morphology in vertebrate hosts</td>
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<td>Monomorphic</td>
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<tr>
<td>Site of infection in vert. hosts</td>
<td>Vascular and extravascular</td>
<td>Vascular; rarely extravascular</td>
</tr>
<tr>
<td>Pathogenicity</td>
<td>Very pathogenic for humans; mildly or nonpathogenic for cattle, pigs, sheep</td>
<td>Very pathogenic for cattle, sheep and pigs; nonpathogenic for humans</td>
</tr>
<tr>
<td>Other differences</td>
<td>C.N.S. invasion; encephalitis; possible immunopathologic mechanisms;</td>
<td>No C.N.S. involvement; no encephalitis; no immunopathologic mechanisms reported;</td>
</tr>
<tr>
<td>plus</td>
<td>Differences in cellular morphology and development in the tsetse fly, drug resistance, and cellular metabolism</td>
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</tr>
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1 Adapted from Soulsby (1968) and Baker (1969).
Experimental animals. Adult male and female albino rabbits, 2.5 - 3.5 kg body weight, were used for experimental infection in this investigation. The animals were caged separately and had food and water available ad libitum. In addition, albino rats (0.2 - 0.5 kg) for passage of trypanosomes and albino guinea pigs (1.0 - 1.5 kg) for skin reactive factor tests were used; these animals were caged in groups of up to ten, and they had food and water available at all times.

The trypanosomes. The strain of *Trypanosoma congo-lense* used in this investigation was provided by Dr. Frans C. Goble (CIBA Pharmaceutical Co.). Maintenance of the strain was accomplished by the intraperitoneal injection of albino rats with liquid nitrogen-preserved infected rat blood, and by the subsequent serial passage of infected blood through rats until death occurred regularly at 48 hr after infection (Figure 5). Heparinized blood was taken by cardiac puncture from termin-
FIGURE 5

Stabilization of the infective *Trypanosoma congolense* strain in liquid nitrogen, and inoculation of rabbits.
LIQUID NITROGEN

PASSAGE OF INFECTED BLOOD 48 HR

RABBIT

TEST FOR INFECTIVITY

5 X 10^5 TRYPANOSOMES IP

DEATH 48 HR
ally ill rats for liquid nitrogen storage and rabbit inoculation. The number of trypanosomes per ml was determined by using a hemacytometer and Kolmer's counting fluid (Kolmer, 1915). One ml aliquots of this trypanosome-containing blood were put into liquid nitrogen storage (Herbert et al., 1968). The remaining blood was diluted with pyrogen-free normal saline to contain approximately $5 \times 10^6$ trypanosomes/ml, and 1 ml of diluted rat blood was injected intraperitoneally into normal rabbits to induce infection. The stabilization of a trypanosome strain in frozen storage, and the passage of the strain in a species other than that used for the investigation fulfills several of Brown's (1967) requirements for immunological studies of antigenically variable trypanosomes.

**Course of infection.** Trypanosome infections were permitted to progress for six weeks after inoculation. The presence of trypanosomes in peripheral blood, weight loss, hematocrit values and gross pathology were noted at regular intervals throughout the infections. A curative dose of homidium bromide (Boots Pure Drug Co. Ltd.) was administered at six weeks; the dosage used was 10 mg/kg
body weight given subcutaneously as a 1% solution on two consecutive days. Homidium bromide is selectively lethal for the parasites in the host, and acts by inhibiting trypanosome DNA replication, RNA synthesis and protein formation (Newton, 1970). After cure, as evidenced by regression of lesions, several ml of heparinized rabbit blood were injected intraperitoneally into rats as a test for possible remaining infection (Figure 5).

Preparation of tissue antigens. Tissues from normal and recovered rabbits (six weeks after homidium bromide treatment) were taken aseptically and immediately frozen in liquid nitrogen for storage at -68° C. Brain, liver, heart and kidney were routinely collected in this manner, and were thawed for extraction just before testing. Test antigen was prepared from each tissue as shown in Figure 6: triethanolamine-buffered saline, TBS, pH 7.2, as prepared by the Department of Serology, Walter Reed Army Institute of Research (Fife, 1971, personal communication) with concentrations of Mg++ and Ca++ optimal for complement fixation (CF) test was added to thawed tissue as 5 ml/gm of tissue; the mixture was homogenized at 4° C in a Waring blender at full speed for
FIGURE 6

Preparation of the CF test antigens from rabbit tissues.
Tissue

TBS pH 7.2 (5ML/GM)
4° C
WARING BLENDER

CRUDE EXTRACT

5,000 x G
15 MIN
4° C

SEDIMENT   SUPERNATE

THIMEROSAL 1:10,000
-68° C

TEST ANTIGEN
3 min; the crude extract was then centrifuged at 5000 X G for 15 min at 4° C, and the supernatant fluid was tested for protein concentration by biuret analysis (Campbell et al., 1964); the fluid was diluted, if necessary, to approximate the protein concentration values given in Table 10; the supernatant fluids thus obtained were designated the CF test antigens; and, the test antigens were preserved with Thimerosal (Lilly) diluted to a final concentration of 1:10,000 and were stored at -68° C until used.

Collection of sera. Blood was taken by cardiac puncture from rabbits just before inoculation with trypanosomes, and at weekly intervals thereafter until twelve weeks post-inoculation (six weeks after homidium bromide treatment). The blood was allowed to clot at room temperature for 1 hr and then was placed at 4° C overnight; sera were separated from clots and centrifuged at 5000 X G for 15 min to remove any contaminating erythrocytes or trypanosomes. Normal and infected rabbit sera were occasionally taken for cell culture media or passive injection procedures. These sera were collected in the same manner but were filter-sterilized with a
Seitz pressure filter apparatus. All sera were stored at -20°C until used.

**Test for complement fixing (CF) antibody to normal rabbit tissues.** All sera taken before, during and after infection were heat-inactivated at 56°C for 30 min. Following treatment, sera were tested for CF activity with normal allogeneic or autologous tissue antigens. The CF procedure was an adaptation of the technique used by Kent and Fife (1963) and the Department of Serology, Walter Reed Army Institute of Research (Fife, 1971, personal communication). Briefly, guinea pig complement (Grand Island Biological Co.) was spectrophotometrically standardized to contain 5 complement 50% hemolytic units (5C'H$_{50}$) per 0.3 ml TBS; and, anti-sheep erythrocyte hemolysin (Grand Island Biological Co.) was similarly standardized for optimal sensitization of sheep erythrocytes (SRBC). The SRBC suspensions were spectrophotometrically standardized to contain 5 X $10^8$ cells/ml TBS. Initially, each tissue antigen and serum sample was serially diluted in buffer and reacted with 2.5C'H$_{50}$/0.3 ml TBS to determine levels of anticomplementarity; the maximum concentration of antigen or serum used in tests
was one two-fold dilution beyond that dilution allowing 100% lysis of hemolysin-sensitized SRBC. For the actual test, two-fold dilutions of sera were prepared in 0.3 ml volumes of TBS. Test antigen and 5C'H50 in 0.3 ml TBS were added to each tube resulting in a final volume of 0.9 ml per tube, and appropriate antigen, serum, complement and erythrocyte controls were included in each test. Tubes were incubated at 4° C for 16 - 18 hr. Following incubation, 0.6 ml of an optimally-sensitized SRBC suspension was added to each tube and all tubes were incubated at 37° C for 30 min. Tubes were then centrifuged at 1000 X G for 1 min and the supernatant fluids were compared to previously prepared standards simulating varying degrees of hemolysis. Serum titers in this CF procedure were based on an endpoint expressed as the reciprocal of that serum dilution giving 50% hemolysis in the presence of tissue antigen, 5C'H50 and optimally-sensitized SRBC.

Mercaptoethanol sensitivity of autoantibody. Duplicate serum samples from six week infected rabbits were diluted 1:4 in TBS. One ml of one sample was added to an equal volume of 0.2M 2-mercaptoethanol (ME) in TBS and 1 ml of the remaining sample was added to 1 ml of TBS
without ME. Both samples were incubated at 37° C for 30 min. Immediately after incubation, the ME-treated serum was dialyzed against several volumes of TBS in a Diaflo cell (Amicon, Inc.) with an XM-50 membrane and then concentrated to the same dilution as the control serum. Both serum samples were then tested for CF activity with normal allogeneic brain and liver antigens.

Fractionation of sera by gel filtration. Sephadex G-200 regular gel (Pharmacia Fine Chemicals, Inc.) was swollen in excess buffer under optimal conditions in the usual manner (Pharmacia, 1970); the buffer used was 0.15M phosphate-buffered saline (PBS), pH 7.4, with 0.02% sodium azide added as a preservative. After swelling, the Sephadex slurry was deaerated under negative pressure and carefully packed into a Pharmacia K25/45 column to within 2 inches of the top. A sample applicator was inserted to protect the upper surface of the gel bed. Before fractionating serum on the column, 1 ml of 1% Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.) was passed through the gel to determine the void volume and bed homogeneity. For the actual fractionation procedure, 1 ml of pooled serum from normal or six week infected rabbits was layered onto the gel and passed through the
column under an operating pressure of 150 mm of water (the distance from the column tubing outlet to the bottom of the air inlet tube of the PBS reservoir). Continuous 2 ml effluent fractions were collected in a CCA model R refrigerated fraction collector (Chromatography Corporation of America), and the effluent stream was monitored at 280 nm with an LKB Uvicord II UV analyzer equipped with an LKB recorder. Fluid corresponding to each peak was collected and dialyzed against TBS to the original serum sample volume with a Diaflo cell (XM-50 membrane); these fractions were analyzed for immunoglobulin content by gel diffusion tests and immunoelectrophoresis with antisera monospecific for IgG, IgA and IgM (Miles Laboratories, Inc.), and were tested for CF activity with normal allogeneic brain and liver antigens.

Separation of 7S and 19S immunoglobulins by density gradient ultracentrifugation. Williams and Chase (1968) present a well-documented procedure for the separation of IgG from IgM by centrifugation in a sucrose gradient. Linear gradients of 10-40% (w/v) sucrose in 0.15M NaCl were formed, using an Isco model 570 gradient former, in 1/2" X 2" cellulose nitrate tubes (Beckman) to a final volume of 4.5 ml per tube. Normal and six week infected
rabbit serum samples were diluted 1:4 in 0.15M NaCl and layered onto the sucrose in 0.5 ml volumes; a Spinco SW-39L rotor containing the tubes was spun at 4°C in a Beckman L2-65B ultracentrifuge for 18 hr at 35,000 rpm. Following centrifugation, the contents of the tubes were fractionated on an Isco density gradient fraction collector using a 48% sucrose "chase" solution. Effluent from each tube was analyzed at a wavelength of 254 nm with an Isco model 222 UV analyzer, and the absorbance peaks were recorded on an Isco model 610 recorder. Fluid corresponding to each peak was collected and dialyzed against TBS to the original serum sample volume with a Diaflo cell (XM-50 membrane); these fractions were analyzed for immunoglobulin content by gel diffusion and immunoelectrophoresis with antisera monospecific for IgG and IgM, and were tested for CF activity with brain and liver antigens.

**Immunoelectrophoretic analyses.** Serum fractions obtained from gel filtration and density gradient ultracentrifugation were tested for immunoglobulin content and for electrophoretic mobility in comparison with whole serum. Twenty ml barbitol-buffered agar, pH 8.6, (Campbell et al., 1964) was melted and poured onto glass
slides contained in an LKB slide frame. When cool, the agar-covered slides were punched with an LKB gel punch in the desired pattern (Figure 12); whole serum and serum fractions were added to the wells and the slide frame was placed in an LKB electrophoresis apparatus. The electrode vessels were filled with 700 ml barbitol buffer and were connected to the slide frame with moist rayon wicks in the usual manner (LKB, 1962). A potential of 250 V at 50 mA was induced across the slides for 45 min. After electrophoresis, the central trough was cut from each slide and the appropriate antiserum introduced. All slides were incubated for 24 hr at room temperature, and for any additional periods of time at 4° C. Resulting patterns were photographed in a Cordis Immunodiffusion Camera on Polaroid type 107 black and white film.

**Gel diffusion tests.** Microimmunodiffusion analyses of infected rabbit sera with trypanosome extracts and of serum fractions with monospecific antisera for immunoglobulins G, A and M were performed. Ionagar No. 2 (0.85%, Consolidated Laboratories, Inc.) was prepared in borate-buffered saline, pH 8.4, according to the method of Campbell et al. (1964). Four ml melted agar were
transferred to precleaned 2.5 X 7.5 cm glass slides and allowed to cool. The agar was then punched in the pattern desired (Figures 13 and 18) with an LKB gel punch, and the reagents were added to the wells in the agar. The slides were incubated for 24 hr at room temperature, and for any additional periods of time at 4° C. Results were photographed in a Cordis Immunodiffusion Camera on Polaroid type 107 black and white film. Gel diffusion tests of infected rabbit sera with tissue antigens were also performed. Petri dishes (100 X 15 mm) were filled with 20 ml of borate-buffered Ionagar No. 2, and wells 5 mm in diameter were punched in the solidified gel; distances between adjacent wells were 2-4 mm. Wells were filled with 0.1 ml of tissue antigen or serum, and the plates were incubated at room temperature for 24 hr and then at 4° C for one week.

**Test for IgM levels in infected rabbit serum.** Four ml amounts of melted Ionagar No. 2 containing monospecific anti-IgM (1/25) were transferred to clean 2.5 X 7.5 cm glass slides. When cool, wells were punched in the agar and equal volumes of pooled sera from normal, infected and recovered rabbits were placed into separate wells.
The slides were incubated at room temperature for 48 hr; the zones of precipitation were photographed with a Cordis Immunodiffusion Camera on Polaroid type 107 black and white film. Serum IgM concentrations were determined by comparison of the diameters of the zones of precipitation of infected and recovered rabbit sera to normal serum and to standards containing known amounts of purified rabbit IgM (Fahey and McKelvey, 1965).

Passive transfer of autoantibody. Pooled serum from infected rabbits was injected into normal rabbits in an attempt to reproduce the pathology observed during the course of infection. Twenty-five ml of serum were injected intraperitoneally, 5 ml were injected subcutaneously on the sides of the rabbits and several intradermal injections of 0.1 ml were given on the back. Normal serum served as a control. All animals were watched closely for several weeks for any symptoms of disease such as edema of the face, ears and extremities, weight loss, necrotic lesions or death. In addition, volumes of pooled normal or infected rabbit serum were mixed with equal volumes of the various tissue extracts; these mixtures were injected intradermally in 0.1 ml amounts into the shaved skin of
normal rabbits. The sites of injection were observed at 4, 8, 24, and 48 hr for any evidence of a skin reaction.

**Absorptions of infected rabbit sera with trypanosome extracts and tissues.** Pooled serum samples from infected rabbits were diluted 1:8 in TBS. Equal volumes of diluted sera and trypanosome extract (2.0 mg protein/ml saline) or small pieces of washed brain and liver tissue in saline were incubated at 37°C for 30 min and then at 4°C overnight. The absorbed samples were centrifuged at 12,000 X G for 30 min at 4°C. The supernatant fluids from these tubes were again absorbed with trypanosome extract or tissues and were tested for CF activity with normal allogeneic brain and liver antigens and trypanosome extract. Results were compared with unabsorbed control sera which had been similarly treated.

**V.D.R.L. tests.** Sera from normal and infected rabbits were examined for V.D.R.L. reactivity to determine if Wasserman antibody was present. Four-tenths ml of the V.D.R.L. antigen (Lederle Laboratories) was added to an equal volume of buffered saline diluent, and the mixture was rotated. An additional 2.6 ml buffered saline was added and this mixture was shaken fifty times within
15 sec. For the test, 0.03 ml of serum from normal or infected rabbits was added to 0.01 ml of the V.D.R.L. antigen mixture on a slide and rotated for 4 min at approximately 160-180 rpm. Slides were then read microscopically at 40 X for flocculation. A saline control and a positive control (known syphilitic serum) were included.

**CF tissue antigen analyses.** Normal rabbit tissue antigens were tested for antigenic "strength" and protein concentration. Antigenic strength was determined for each tissue extract by diluting the extract serially in TBS and testing for CF reactivity with a standard dilution (1/32) of pooled serum from infected rabbits. Protein concentration was determined by biuret analysis at the time of extraction. Bovine serum albumin was used as a standard.

**Preparation of tissue antigen for use in cellular sensitivity tests and skin tests.** The extraction procedure for preparation of the complement fixation test antigens (Figure 6) was used, with the differences listed below. Only normal allogeneic brain was used. Pyrogen-free saline or Minimal Essential Medium and Minimal Essential Medium-Suspension Culture (MEM and MEM-S,
Grand Island Biological Co.), pH 7.2 with 15% allogeneic normal rabbit serum, 200mM L-glutamine and 100 U/ml penicillin G were used in a 1:1 w/v ratio instead of TBS. A hand tissue homogenizer was used instead of a Waring blender in order to minimize foaming. The final supernatant fluid to be used as the test antigen was force-filtered through a Swinney filter (Millipore membrane, 0.45 µm pore size) into sterile vaccine bottles without the addition of Thimerosal, and the brain antigen concentrate was diluted in fresh saline, MEM or MEM-S on the day of testing.

Preparation of trypanosome extracts for use in cellular sensitivity tests and skin tests. The method of Lanham and Godfrey (1970) was adopted for the separation of trypanosomes from whole blood. Briefly, DEAE-cellulose (Celusorb DEAE, General Biochemicals) was suspended in excess buffer for equilibration, and then washed an additional four times with buffer; the buffer used was phosphate-buffered saline-glucose (PSG, pH 8.0 with 1% glucose and an ionic strength of 0.217). After the final wash, the pH of the equilibrated DEAE-cellulose slurry was checked and adjusted with 5% H₃PO₄ to pH 8.0. The
slurry was then frozen at -20°C until used, at which time it was rewashed with five volumes of fresh PSG.

Rats were infected with liquid nitrogen cultures of trypanosomes or with trypanosomes in the blood of rabbits with two, four and six week infections. Infected rats with high parasitemias were bled by cardiac puncture and their heparinized blood was mixed 1:3 with cold buffer. The thawed, washed DEAE-cellulose was poured into a 250-20 Chromaflex column (Kontes Glass Co.) with a Chromaflex 20 adapter containing a sintered glass disc and Whatman No. 42 filter paper (W. and R. Balston, Ltd.). PSG was run through the column until the buffer meniscus met the packed cellulose; 30 ml diluted rat blood was carefully added and allowed to pass slowly through the column. The eluate was constantly monitored for trypanosomes, which were collected well before the erythrocytes and other blood cells were eluted from the column. The trypanosomes were centrifuged at 1800 X G for 20 min, washed twice in fresh PSG and then osmotically lysed according to the procedure of Mahmoud (1970). The lysates were pooled, lyophilized and then reconstituted in pyrogen-free saline, MEM or MEM-S to form solutions of various protein
concentrations. Protein concentrations were determined by biuret analysis. The reconstituted crude trypanosome extract was tested for precipitation in gel with sera from normal, infected and recovered rabbits. The crude trypanosome extract was also arbitrarily separated into fractions for skin tests by means of a Diaflo cell containing an XM-50 membrane. The fractions were the filtrate, predominantly containing molecules of $\text{MW} < 5 \times 10^4$, and the retentate, containing most molecules of $\text{MW} > 5 \times 10^4$. All rehydrated crude trypanosome extracts or fractions were stored at $-20^\circ\text{C}$ until used.

Skin tests. Normal, infected and recovered rabbits were skin-tested with various concentrations of trypanosome extract and brain antigen prepared in pyrogen-free normal saline. The crude trypanosome extract injections contained 0.8, 0.4 and 0.04 mg protein/0.1 ml saline. An injection of trypanosome extract fraction (most molecules of $\text{MW} > 5 \times 10^4$) contained 0.2 mg protein/0.1 ml saline and a fraction containing molecules predominantly of $\text{MW} < 5 \times 10^4$ contained 0.04 mg protein/0.1 ml. Brain antigen injections contained 0.8, 0.4 and 0.08 mg protein/0.1 ml saline. All rabbits received 0.1 ml of the antigens
intradermally on their shaved sides. Results of the skin tests were read at 4, 8, 18, 24, 48 and 72 hr by gauging the area of induration with calipers and by making visual estimations of erythema and necrosis.

**Passive transfer of skin reactions to normal rabbits.**
In an attempt to passively transfer the Arthus reactions to normal rabbits, 1 ml of trypanosome extract (8.0 mg protein/ml saline) was mixed and incubated with an equal volume of infected rabbit serum at room temperature for 30 min; 0.1 ml of this mixture, containing reactants in the zone of antigen excess for a precipitation reaction, was injected intradermally into the shaved skin of normal rabbits. Controls consisted of intradermal injections of normal rabbit serum mixed with trypanosome extract and of trypanosome extract (0.4 mg protein/0.1 ml saline), infected and normal rabbit serum separately. All sites of injection were observed at 4, 8, 18, 24, 48 and 72 hr post-inoculation. Induration and erythema were gauged at each observation.

**Histological analyses of skin test reactions and infected rabbit organs.** Multiple skin reactions were induced in an infected rabbit by intradermal injections of
crude trypanosome extract (0.4 mg protein/0.1 ml saline). Biopsies were made on 4, 8, 24 and 48 hr skin reaction sites as well as on skin receiving 0.1 ml saline. The tissue sections were washed free of blood with physiological saline and fixed in 10% neutral formalin for at least 48 hr. Samples of brain, liver, heart, kidney and spleen were also collected from normal and infected rabbits. All tissues were washed, dehydrated, infused with and embedded in paraffin in the usual manner (Humason, 1967); embedded tissues were sectioned 7 μm thick with an A. O. Spencer microtome, mounted on slides and stained with hematoxylin and eosin (Humason, 1967). Skin test reaction sections were examined for cellular infiltration. The other tissues were examined for cellular infiltration, trypanosomes and any other differences from normal rabbit tissues.

Test for migration inhibitory factor (MIF). The procedure used for detection of MIF was derived from techniques outlined by David et al. (1964a and 1964b), Bloom and Bennett (1971) and Thor (1971). Normal and infected rabbits were given 50 ml Marcol 52 (light mineral oil, Humble Oil and Refining Co.) intraperitoneally two days prior to collection of peritoneal exudate cells (PEC).
On the day of collection, rabbits were exsanguinated by cardiac puncture, the abdominal wall muscle layer was aseptically exposed and 50 ml Hanks balanced salt solution (HBSS) containing 10 U/ml heparin was injected intraperitoneally; the heparin-HBSS solution was massaged throughout the peritoneal cavity. An incision was then made in the abdominal wall through which a sterile perforated collecting tube was inserted. The PEC were aseptically transferred to sterile 60 ml separatory flasks in which the aqueous layer containing the cells was separated from the oil into sterile centrifuge tubes. The PEC were centrifuged from the aqueous layer at 50 X G for 10 min, washed twice in HBSS, counted and resuspended in a volume of MEM to give approximately 5 X 10^7 viable PEC/ml. Sterile capillary tubes were filled with PEC from normal and infected rabbits, plugged with sterile clay and centrifuged in a sterile hematocrit head at 50 X G for 2 min. The capillary tubes were then scored and broken at the cell-medium interface, and placed into sterile Sykes-Moore chambers; sterile silicone vacuum grease (Beckman) was used to hold the cell-packed tubes in position. The chambers were sealed and filled with MEM containing allogeneic brain antigen (4.0 and 0.4 mg protein/ml) or
trypanosome extract (2.0 and 0.2 mg protein/ml). Controls consisted of the chambers with normal PEC, chambers filled with MEM containing no antigen, and a positive control. The positive controls were PEC that had been incubated with 25 μg/ml Concanavalin A (Jackbean phytohemagglutinin, Calbiochem) in MEM for 30 min at 37°C and then washed twice before being packed into capillary tubes and incubated in MEM. All samples were done in triplicate, and the chambers were incubated for 24 hr at 37°C. After incubation, the areas of PEC migration were determined from photographic enlargements of the test chambers. Experimental PEC migration values were compared to control values, and the Migration Index (MI) calculated (David et al., 1964a):

\[ MI = \frac{\text{Ave. area PEC migration with antigen}}{\text{Ave. area PEC migration without antigen}} \]

Test for skin reactive factor (SRF). The following SRF procedure was adapted from Schwartz et al. (1970) and Bloom and Bennett (1971). Spleens were removed from the normal and infected rabbits used in the MIF studies and placed into sterile petri dishes containing HBSS. The spleen cells (SPC) were teased apart with dissecting
needles and strained through several layers of sterile gauze into centrifuge tubes. SPC were spun at 50 X G for 10 min, washed twice in HBSS, counted and resuspended in MEM-S to give approximately $5 \times 10^8$ viable SPC/ml. One ml of the SPC inoculum was added to 2.0 ml MEM-S containing brain antigen (0.4 mg protein/ml) or trypanosome extract (0.2 mg protein/ml) in 12 X 75 mm sterile plastic tubes (Falcon Plastics). Negative controls consisted of normal SPC cultures, SPC incubated in MEM-S without antigen, and positive controls consisted of SPC stimulated with 15% fetal calf serum in MEM-S. All cells were incubated for 24 hr at 37° C in an atmosphere of 5% CO$_2$ in air. Following incubation, SPC were centrifuged at 100 X G for 10 min and the culture supernatant fluids were removed to storage at -20° C. On the day of testing, the culture fluids were thawed and injected intradermally in 0.1 ml amounts into normal rabbits and guinea pigs. The sites of injection were observed for erythema and induration every 4 hr up to 24 hr, and then again at 48 hr; experimental sites were compared with control sites. Areas of induration more than twice the size of those produced by the negative control injections were considered significant.
RESULTS

Course of infection. Infected rabbits developed edematous sites about the face, ears and extremities after about two weeks of infection; some rabbits were unable to hold their edematous ears erect. Edema progressed to necrosis at about three to four weeks (Figure 7). The necrotic areas were primarily located on the ears, about the eyes and nose, on appendages and the genitals. Lesions increased in intensity until cure with homidium bromide or death (Figure 8). Throughout the infections, slight reductions in weight and hematocrit values were noted (Table 1). Few or no trypanosomes were observed in the animals' blood at any time during infection, although blood in volumes of several ml was infective for rats. Several rabbits died before chemotherapy was instituted; these animals displayed no greater external pathology at the time of death than the surviving animals. Most organs of infected rabbits appeared normal upon macroscopic examination, but grossly enlarged spleens
FIGURE 7

Necrotic lesions on the face and ears of a rabbit infected with *Trypanosoma congoense*.
(Four weeks post-inoculation)
FIGURE 8

Necrosis of the face, ears and genitals of a Trypanosoma congolense-infected rabbit at death.
(Six weeks post-inoculation)
TABLE 1

Mean weights and hematocrit values of infected rabbits.

<table>
<thead>
<tr>
<th>Time</th>
<th>Mean Weights (kg)</th>
<th>Mean Hematocrit Values (% packed cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>3.0</td>
<td>38</td>
</tr>
<tr>
<td>Infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>2.8</td>
<td>33</td>
</tr>
<tr>
<td>Week 2</td>
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<td>Week 3</td>
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<td>Week 5</td>
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<td>Week 6</td>
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<tr>
<td>Post-infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 8</td>
<td>2.8</td>
<td>35</td>
</tr>
<tr>
<td>Week 10</td>
<td>2.9</td>
<td>37</td>
</tr>
<tr>
<td>Week 12</td>
<td>3.1</td>
<td>38</td>
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(up to five times normal size) and occasional cortical congestion of the kidneys were observed. Homidium bromide treatment resulted in complete recovery of the rabbits: blood was no longer infective for rats, weight was gained and the lesions healed rapidly within one to two weeks. Histological analyses of the brain, liver, heart or kidney of infected rabbits revealed no mononuclear cell infiltration. No microscopic pathology was observed in any of the organs except kidney where glomeruli and tubules were damaged and were sometimes filled with an eosinophilic substance. No trypanosomes were observed in the sections.

**CF reactivity of infected rabbit sera with tissue antigens.** The different tissue antigens displayed various levels of anticomplementary effects when reacted with $2.5C'H_{50}$, but each preparation of test antigen from each tissue was usually anticomplementary to the same dilution. For the CF tests, brain was diluted to 1/4, liver to 1/32 and heart and kidney to 1/64; at these dilutions, the antigens did not bind complement nonspecifically. Infected rabbit sera were usually anticomplementary to 1/4 or 1/8 dilutions; as a consequence, all sera were tested at
dilutions of 1/8 and higher. During infection some serum samples were anticomplementary to dilutions of 1/32. Most rabbits exhibited low titers (8 to 64) of complement-fixing antibody to autologous or normal allogeneic tissue antigens prior to infection. All rabbits developed antibody (up to titers of 512) to the tissue antigens after infection. The antibody responses were greatest to brain and were less to liver, heart and kidney. Five rabbits' sera (40, 41, 42, 43, 44) had the same titers with autologous tissues as with normal allogeneic tissues. CF titers fell to preinfection levels after homidium bromide treatment. Antigen, serum and complement controls were uniformly negative throughout the series of tests; erythrocyte controls revealed no spurious lysis. All results are presented in Tables 2 to 6. The mean complement-fixing antibody responses to normal allogeneic tissues are depicted diagramatically in Figure 9.

Mercaptoethanol sensitivity of autoantibody. CF activity of pooled six week infected rabbit serum with tissue antigens was reduced by ME treatment (Table 7).
<table>
<thead>
<tr>
<th>Sera</th>
<th>Weeks After Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>R34</td>
<td>16</td>
</tr>
<tr>
<td>R35</td>
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</tr>
<tr>
<td>R36</td>
<td>&gt;16</td>
</tr>
<tr>
<td>R37</td>
<td>32</td>
</tr>
<tr>
<td>R38</td>
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</tr>
<tr>
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<td>64</td>
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<td>R41</td>
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<td>R43</td>
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<td>R44</td>
<td>32</td>
</tr>
<tr>
<td>R45</td>
<td>32</td>
</tr>
<tr>
<td>R46</td>
<td>16</td>
</tr>
</tbody>
</table>

Titer is expressed as the reciprocal of that dilution of antiserum giving 50% hemolysis with $5C'_{50}$ units and optimally-sensitized SRBC in the presence of antigen.

* Trypanocidal drug treatment.
TABLE 3

CF activity of sera from *Trypanosoma congolense*-infected rabbits with normal allogeneic liver antigen

<table>
<thead>
<tr>
<th>Sera</th>
<th>Titers Against Liver Antigen&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Weeks After Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 1 2 3 4 5 6* 7 8 9 10 11 12</td>
</tr>
<tr>
<td>R34</td>
<td></td>
<td>8 32 64 128 128 128 128 32 32 8 16 8 8</td>
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<tr>
<td>R35</td>
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</tr>
<tr>
<td>R36</td>
<td></td>
<td>16 64 128 Death</td>
</tr>
<tr>
<td>R37</td>
<td></td>
<td>32 64 128 128 256 256 256 128 128 64 32 32 32</td>
</tr>
<tr>
<td>R38</td>
<td></td>
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<td>32 256 256 512 512 512 512 512 256 128 64 32 32</td>
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<td>32 128 256 256 256 256 256 256 256 128 64 32 32</td>
</tr>
<tr>
<td>R41</td>
<td></td>
<td>32 64 128 128 Death</td>
</tr>
<tr>
<td>R42</td>
<td></td>
<td>16 32 128 128 128 128 64 64 16 16 16 16 16</td>
</tr>
<tr>
<td>R43</td>
<td></td>
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<td>R44</td>
<td></td>
<td>32 128 128 256 128 256 256 128 64 32 32 32 32</td>
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<tr>
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<td></td>
<td>16 64 128 128 256 256 256 128 64 64 32 16 16 16</td>
</tr>
<tr>
<td>R46</td>
<td></td>
<td>32 64 128 256 256 256 Death</td>
</tr>
</tbody>
</table>

<sup>1</sup> Titer is expressed as the reciprocal of that dilution of antiserum giving 50% hemolysis with 5C<sub>100</sub> units and optimally-sensitized SRBC in the presence of antigen.

* Trypanocidal drug treatment.
TABLE 4

CF activity of sera from *Trypanosoma congolense* -infected rabbits with normal allogeneic heart antigen

<table>
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<tr>
<th>Sera</th>
<th>Weeks After Inoculation</th>
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</thead>
<tbody>
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</tr>
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<td>R34</td>
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<td>R40</td>
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<td>R41</td>
<td>8</td>
</tr>
<tr>
<td>R42</td>
<td>NT</td>
</tr>
<tr>
<td>R43</td>
<td>NT</td>
</tr>
<tr>
<td>R44</td>
<td>NT</td>
</tr>
<tr>
<td>R45</td>
<td>8</td>
</tr>
<tr>
<td>R46</td>
<td>8</td>
</tr>
</tbody>
</table>

1 Titer is expressed as the reciprocal of that dilution of antiserum giving 50% hemolysis with 5C'H₅₀ units and optimally-sensitized SRBC in the presence of antigen. NT = no titer; greater than 50% hemolysis observed with serum diluted 1/8.

* Trypanocidal drug treatment.
### TABLE 5

CF activity of sera from *Trypanosoma congoense*-infected rabbits with normal allogeneic kidney antigen

<table>
<thead>
<tr>
<th>Sera</th>
<th>Weeks After Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>R34</td>
<td>NT</td>
</tr>
<tr>
<td>R35</td>
<td>NT</td>
</tr>
<tr>
<td>R36</td>
<td>NT</td>
</tr>
<tr>
<td>R37</td>
<td>NT</td>
</tr>
<tr>
<td>R38</td>
<td>NT</td>
</tr>
<tr>
<td>R39</td>
<td>NT</td>
</tr>
<tr>
<td>R40</td>
<td>8</td>
</tr>
<tr>
<td>R41</td>
<td>8</td>
</tr>
<tr>
<td>R42</td>
<td>NT</td>
</tr>
<tr>
<td>R43</td>
<td>NT</td>
</tr>
<tr>
<td>R44</td>
<td>NT</td>
</tr>
<tr>
<td>R45</td>
<td>NT</td>
</tr>
<tr>
<td>R46</td>
<td>NT</td>
</tr>
</tbody>
</table>

1. Titer is expressed as the reciprocal of that dilution of antiserum giving 50% hemolysis with 5C’H₅₀ units and optimally-sensitized SRBC in the presence of antigen. NT = no titer; greater than 50% hemolysis observed with serum diluted 1/8.

* Trypanocidal drug treatment.
**TABLE 6**

Mean log₂ CF titers and ranges of normal, peak infected and recovered rabbit sera with allogeneic tissue antigens

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Normal Sera</th>
<th>Infected Sera¹</th>
<th>Recovered Sera³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>4.5 (4-6)</td>
<td>8.6 (8-9)</td>
<td>5.2 (4-6)</td>
</tr>
<tr>
<td>Liver</td>
<td>4.6 (3-6)</td>
<td>7.8 (7-9)</td>
<td>4.7 (3-6)</td>
</tr>
<tr>
<td>Heart</td>
<td>&lt;3 (NT-3)</td>
<td>5.3 (5-6)</td>
<td>&lt;3 (NT-3)</td>
</tr>
<tr>
<td>Kidney</td>
<td>&lt;3 (NT-3)</td>
<td>4.8 (4-6)</td>
<td>&lt;3 (NT-3)</td>
</tr>
</tbody>
</table>

¹ Mean titer is expressed as the mean log₂ value of the reciprocal of that dilution of antiserum giving 50% hemolysis with 5C'H₅₀ units and optimally-sensitized SRBC in the presence of antigen. NT = no titer.

² Mean titers at peak CF reactivity with tissue antigens.

³ Mean titers six weeks after cure.
FIGURE 9

Mean log₂ CF titers of sera from *Trypanosoma congolense*-infected rabbits with normal allo­
geneic tissue antigens.
WEEKS AFTER INFECTION

- BRAIN
- LIVER
- HEART
- KIDNEY

MEAN LOG$_2$ TITER
TABLE 7

CF reactivity of pooled infected rabbit serum treated with 0.2M 2-mercaptoethanol

<table>
<thead>
<tr>
<th>Serum</th>
<th>Titer(^1) against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Mercaptoethanol-treated</td>
<td>32</td>
</tr>
<tr>
<td>Untreated control</td>
<td>256</td>
</tr>
</tbody>
</table>

\(^1\) Titer is expressed as the reciprocal of that dilution of antiserum giving 50% hemolysis with 5C'H\(_{50}\) units and optimally sensitized SRBC in the presence of antigen.
Gel filtration analyses. Pooled normal and infected rabbit sera exhibited three protein peaks (Figures 10 and 11). Fraction 1, peak 1 was in the void volume of the Sephadex G-200 fractionated sera; CF activity with brain and liver antigens occurred only in Fraction 1 of infected rabbit serum (Figure 11). Gel diffusion and immunoelectrophoretic analyses of the protein peak fractions revealed that Fraction 1 contained IgM, Fraction 2 had IgG and trace amounts of IgA and Fraction 3 had some IgG (Figures 12 and 13).

Density gradient ultracentrifugation. Serum samples from normal and infected rabbits were separated into four protein-containing fractions by density gradient ultracentrifugation (Figures 14 and 15). Gel diffusion and immunoelectrophoretic analyses revealed that IgG occurred in peak 2 and that IgM was in peak 3 of the protein concentration profiles (Figures 13 and 16). CF activity with tissue antigens occurred only in peak 3 of infected rabbit serum samples, and the activity was as high as that of untreated serum samples from infected rabbits (Figure 15).

Radial immunodiffusion tests for IgM levels. Infected and recovered rabbit sera had IgM levels of up to four times
Absorbance of pooled normal rabbit serum fractionated on a Sephadex G-200 column. Fraction 1 contains IgM and is in the void volume. Fraction 2 contains most IgG and some IgA, and Fraction 3 contains serum albumin and traces of IgG.
FIGURE 11

Absorbance of pooled infected rabbit serum fractionated on a Sephadex G-200 column, and fraction CF reactivity with tissue antigens. Fraction 1 contains IgM and is in the void volume; all CF reactivity with normal rabbit tissues occurs in Fraction 1. Fraction 2 contains most IgG and some IgA, and Fraction 3 has traces of IgG and contains the serum albumin.
Fraction Reactivity (% of Whole Serum Reactivity) vs. Effluent Volume (ml).

Transmission (280 nm)
FIGURE 12

Immunoelectrophoretic analyses of infected rabbit serum Fractions 1 and 2 separated by gel filtration. Precipitation patterns reveal that Fraction 1 contains only IgM, that Fraction 2 contains only IgG and that IgM and IgG are effectively separated by gel filtration.
FIGURE 13

Gel diffusion analyses of Fractions of infected rabbit serum separated by gel filtration and ultracentrifugation.

Center well (1) = monospecific anti-IgM
Center well (2) = monospecific anti-IgG
A = Fraction 1, gel filtration
B = Fraction 3, ultracentrifugation
C = Fraction 2, gel filtration
D = Fraction 2, ultracentrifugation
E = Unfractionated infected rabbit serum
F = Saline control

Precipitation patterns show that IgM was separated from IgG by gel filtration and ultracentrifugation, and that the fractions containing the respective immunoglobulins had molecules of identical antigenicity.
FIGURE 14

Absorbance of normal rabbit serum fractionated by density gradient ultracentrifugation. Fraction 2 contains IgG and Fraction 3 contains IgM.
FIGURE 15

Absorbance of infected rabbit serum fractionated by density gradient ultracentrifugation, and Fraction CF reactivity with tissue antigens. Fraction 2 contains IgG and Fraction 3 contains IgM. All CF reactivity with normal rabbit tissues occurs in Fraction 3.
FIGURE 16

Immunoelectrophoretic analyses of infected rabbit serum Fractions 2 and 3 separated by density gradient ultracentrifugation. Precipitation patterns reveal that Fraction 2 contains only IgG, that Fraction 3 contains only IgM and that IgG and IgM are effectively separated by density gradient ultracentrifugation.
the levels of normal serum; recovered rabbit serum IgM levels were lower than infected rabbit serum levels. Representative results are presented in Figure 17.

**Gel diffusion tests with trypanosome extracts and tissue antigens.** Sera from infected and recovered rabbits contained precipitating antibody to crude trypanosome extracts (Figure 18). Infected rabbit serum also contained precipitating antibody to tissue antigens. Single, diffuse lines of precipitation were observed with brain, heart and kidney antigens; liver antigen gave 2 bands upon occasion. Much diffuse precipitation around antiserum wells, which did not occur when normal serum was used, was also observed. Representative precipitation results are presented in Figure 19.

**Passive transfer of autoantibody.** Normal rabbits that received injections of pooled normal or infected rabbit serum did not die or develop gross external pathology of the type occurring in trypanosome-infected rabbits. No pathology was observed at the sites of injection of pooled sera mixed with the tissue extracts.

**Absorptions of sera.** Absorption of serum from infected rabbits with crude trypanosome extract did not remove CF activity for tissue antigens but did remove
FIGURE 17

Radial immunodiffusion test for IgM concentration of normal rabbit serum (1), three week infected rabbit serum (2), six week infected rabbit serum (3) and recovered rabbit serum (4). Monospecific anti-IgM diluted 1/25 was incorporated into the agar. The precipitation patterns show that IgM concentrations increase in rabbit serum after infection with T. congolense.
FIGURE 18

Gel diffusion test for precipitating antibody to trypanosome antigens. Well (1) contains infected rabbit serum and well (2) has normal rabbit serum. The outer wells contain the crude trypanosome extract at differing distances from the antiserum or normal serum wells. The test demonstrates the presence of antibody to parasite antigens in the serum of infected rabbits. Resolution of the precipitating bands increased with increasing distance of the trypanosome extract from the antiserum well.
FIGURE 19

Gel diffusion tests for precipitating antibody to tissue antigens. I = infected rabbit serum; B = brain antigen; H = heart antigen; K = kidney antigen; L = liver antigen; S = saline. The tests reveal the presence of precipitating auto-antibody in infected rabbit serum to normal tissue extracts.
CF activity for trypanosome extract (Table 8). When infected rabbit sera were absorbed with tissues, however, anticomplementary levels rose so high as to prevent testing of the sera (up to dilutions of 1/256). Enhanced anticomplementarity was presumably due to soluble antigen-antibody complexes in serum.

**V.D.R.L. tests.** Sera from normal and infected rabbits were uniformly V.D.R.L. negative; controls with known syphilitic serum were positive.

**Tissue antigen analyses.** The various tissue antigens had different antigenic strengths. The CF activity with a standard dilution of antiserum decreased in the order liver > heart = kidney > brain (Table 9). Protein concentrations of the test antigens differed, as shown in Table 10. At optimal dilution for CF test, brain and liver had the highest protein concentrations, and heart and kidney had the lowest protein concentrations.

**Skin tests.** Normal, infected and recovered rabbits developed no skin reactions to intradermal injections of brain antigen, trypanosome fractions of MW < 5 X 10⁴ or to saline controls. Skin reactions were observed, however, at the sites of injection with crude trypanosome
### TABLE 8

CF activity of pooled infected rabbit serum absorbed with trypanosome extract

<table>
<thead>
<tr>
<th>Serum</th>
<th>Titer Against</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>Liver</td>
<td>Trypanosome Extract</td>
<td></td>
</tr>
<tr>
<td>Absorbed</td>
<td>256</td>
<td>128</td>
<td>&lt;64</td>
<td></td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>256</td>
<td>128</td>
<td>2048</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>256</td>
<td>128</td>
<td>2048</td>
<td></td>
</tr>
</tbody>
</table>

1. Titer is expressed as the reciprocal of that dilution of antiserum giving 50% hemolysis with $5C'H_{50}$ units and optimally-sensitized SRBC in the presence of antigen.

2. Optimal dilution for CF test was 0.13 mg protein/ml TBS.
### TABLE 9
Antigenic "strength" of allogeneic tissue extracts tested with pooled infected rabbit serum

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Titer $^1$ With Pooled Serum</th>
<th>Ratio of Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>32</td>
<td>1:</td>
</tr>
<tr>
<td>Liver</td>
<td>256</td>
<td>8:</td>
</tr>
<tr>
<td>Heart</td>
<td>128</td>
<td>4:</td>
</tr>
<tr>
<td>Kidney</td>
<td>128</td>
<td>4</td>
</tr>
</tbody>
</table>

$^1$ Titer is expressed as the reciprocal of that dilution of antigen giving 50% hemolysis with 5C$^{H}_{50}$ units and optimally-sensitized SRBC in the presence of a standard dilution (1/32) of antiserum.
**TABLE 10**

Protein concentrations of CF test antigens as demonstrated by the biuret method

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Test Antigen Extract (mg protein/ml)</th>
<th>Optimal Dilution for CF Test (mg protein/ml)</th>
<th>CF Test Protein Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>4.5</td>
<td>1.13</td>
<td>7.1:</td>
</tr>
<tr>
<td>Liver</td>
<td>18.0</td>
<td>0.56</td>
<td>3.5:</td>
</tr>
<tr>
<td>Heart</td>
<td>10.0</td>
<td>0.16</td>
<td>1:</td>
</tr>
<tr>
<td>Kidney</td>
<td>12.0</td>
<td>0.19</td>
<td>1.2</td>
</tr>
</tbody>
</table>
extract (protein concentration ranges of 0.8 to 0.2 mg/0.1 ml saline gave optimal responses) and trypanosome extract fractions of MW > 5 x 10^4 in infected and recovered rabbits. A minimal inflammatory response which was characterized by slight induration (always < 1/6 of the infected rabbit responses) and erythema occurred in normal rabbits injected with the trypanosome extracts. The skin reactions in infected and recovered rabbits developed as erythematous areas of induration the intensity of which increased from about 4 hr after injection to peak reaction at 18-20 hr; erythema and induration decreased steadily after 24 hr. Necrosis frequently developed at the sites as early as 12 hr post-injection and persisted for many days in the infected rabbits. Representative skin reactions in normal and infected rabbits are shown in Figure 20. Histological analyses of the skin reactions following trypanosome extract injections in infected rabbits revealed massive polymorphonuclear leucocyte (PMN) infiltration at 4, 8 and 24 hr while saline-injected sites in the same rabbits showed little change. Necrosis of the epidermis, edema of the dermis, perivascular "cuffing" with PMN's and PMN accumulation within blood vessels were observed in the 4 and 8 hr sections. The cellular infiltration seen in the dermal
FIGURE 20

Skin reactions of normal and Trypanosoma congoense-infected rabbits following intradermal injection of 0.4 mg trypanosome extract protein/0.1 ml saline. The initiation, intensity and duration of the skin response in infected rabbits is indicative of an Arthus reaction.
Induration (mm²)

Hours after injection

Infected

Necrosis

Normal

No necrosis

8 16 24 48 72
and hypodermal layers at 24 hr was predominantly PMN (95%) in nature. Skin sections at 48 hr were almost normal in appearance, except that there was an apparent thickening of the epidermis and an increase in dermal fibroblasts and other monocytic cells. Photographs of the skin sections are presented in Figures 21 through 26.

**Passive transfer of Arthus reactions.** Intradermal injections of infected rabbit serum-trypanosome extract mixtures into normal rabbits produced characteristic Arthus reactions; peak reactions were observed from 12 to 20 hr after inoculation. Normal rabbit serum-trypanosome extract mixtures induced slight reactions in skin which were never greater than 1/4 the size of the infected rabbit serum-trypanosome extract reactions. Separate injections of sera alone gave no skin responses; trypanosome extract injections produced minimal inflammatory reactions characterized by erythema and slight induration.

**MIF tests.** Normal and infected rabbit PEC migrated similarly in the presence of medium, brain antigen and trypanosome extract. Concanavalin A-treated PEC failed to migrate or migrated very little from the capillary tubes. MI values are presented in Table 11 and representative photographs of MIF test migration patterns are
FIGURE 21

Skin sections taken from the site of saline injection in an infected rabbit. Upper photograph shows normal epidermis and dermis; lower photograph depicts normal blood vessels in the dermal layer. (H and E, X 160)
FIGURE 22

Sections of infected rabbit's skin 4 hr after receiving trypanosome extract intradermally. Upper photograph shows intense focal necrosis and PMN accumulation in the epidermis; lower photographs depict PMN accumulation within blood vessels, and perivascular cuffing of PMN's. (H and E, X 160 upper and lower left, X 440 lower right)
FIGURE 23

Sections of infected rabbit's skin 8 hr after receiving trypanosome extract intradermally. Upper photograph shows progressive necrosis of the epidermis and PMN focal accumulation; lower picture shows perivascular cuffing of PMN's. (H and E, X 160)
FIGURE 24

Section of infected rabbit's skin 24 hr after receiving trypanosome extract intradermally. PMN infiltration of dermal and hypodermal layers. (H and E, X 160)
FIGURE 25

Sections of infected rabbit's skin 24 hr after receiving trypanosome extract intradermally. PMN infiltration is predominant, and reveals the Arthus nature of the skin reaction. (H and E, X 440 upper and X 1000 lower)
FIGURE 26

Sections of infected rabbit's skin 48 hr after receiving trypanosome extract. Skin sections are essentially normal in appearance. (H and E, X 160)
TABLE 11

Migration inhibition index (MI) values of normal and infected rabbit peritoneal exudate cells (PEC) incubated in the presence of brain antigen or trypanosome extract (Tryp)

<table>
<thead>
<tr>
<th>MI Values¹</th>
<th>PEC Source</th>
<th>Antigens (mg protein/ml MEM)</th>
<th>Positive Control (µg/ml MEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Brain (4.0)</td>
<td>Brain (0.4)</td>
</tr>
<tr>
<td>Normal Rbt</td>
<td>111</td>
<td>97</td>
<td>114</td>
</tr>
<tr>
<td>Normal Rbt</td>
<td>109</td>
<td>102</td>
<td>103</td>
</tr>
<tr>
<td>Normal Rbt</td>
<td>100</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>100</td>
<td>99</td>
<td>165</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>123</td>
<td>109</td>
<td>132</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>102</td>
<td>112</td>
<td>115</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>107</td>
<td>99</td>
<td>102</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>98</td>
<td>99</td>
<td>106</td>
</tr>
</tbody>
</table>

¹ Expressed as a percentage: MI × 100.
shown in Figure 27.

**SRF tests.** Supernatant fluids from centrifuged normal and infected rabbit SPC cultures containing brain antigen, trypanosome extract or 15% fetal calf serum did not elicit skin reactions in normal rabbits. Guinea pigs responded to supernatant fluids from normal or infected rabbit SPC cultures stimulated by fetal calf serum. All tests were subsequently performed in guinea pigs instead of rabbit, because of the nonreactivity of rabbit skin to supernatant fluids from minimally-stimulated SPC cultures. All results indicate that there is no greater SRF response to brain antigen or to trypanosome extract than to controls containing no antigen (Table 12).
Representative MIF test migration patterns of normal and infected rabbit PEC. BTA = brain tissue antigen (0.4 mg protein/ml MEM); TRYP = trypanosome extract (0.2 mg protein/ml MEM); CON. A = Concanavalin A-treated PEC (25 µg/ml MEM). Migration patterns show that there is no cell-mediated hypersensitivity to normal brain antigen or to trypanosome antigen in T. congolense-infected rabbits.
TABLE 12

Skin reactive factor test in guinea pigs given intradermal injections of supernatant fluids from normal and infected rabbit spleen cell (SPC) cultures incubated with brain antigen or trypanosome extract

<table>
<thead>
<tr>
<th>SPC Source</th>
<th>Cultures</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEM-S</td>
<td>Brain (0.4 mg protein/ml MEM-S)</td>
</tr>
<tr>
<td>Normal Rbt</td>
<td>280</td>
<td>280</td>
</tr>
<tr>
<td>Normal Rbt</td>
<td>315</td>
<td>345</td>
</tr>
<tr>
<td>Normal Rbt</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>240</td>
<td>280</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>315</td>
<td>315</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>530</td>
<td>452</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>280</td>
<td>240</td>
</tr>
<tr>
<td>Infected Rbt</td>
<td>420</td>
<td>400</td>
</tr>
</tbody>
</table>

1 The area of induration at the greatest diameter of induration (mm²).
DISCUSSION

Autoimmunity may occur in infections with various microorganisms. Classical examples are autoantibodies that arise to heart tissue in *Trypanosoma cruzi* and certain streptococcal infections (Kaplan, 1965; Fletcher and Wenger, 1968; Lelchuck et al., 1970), antibody to erythrocytes in *Plasmodium* spp. infections (Zuckerman, 1964; Kreier et al., 1966), autoantibody to brain, lung and erythrocytes in *Mycoplasma pneumoniae* infection (Schmidt et al., 1965; Biberfeld, 1971) and autoantibody to various tissues in viral diseases (Holborow et al., 1963; Allison et al., 1971).

Events related to autoimmunity have been documented in African trypanosomiasis. Enhanced erythrocyte sedimentation rates, spontaneous "autoagglutination" of red cells and adhesion of trypanosomes to erythrocytes, leucocytes and platelets have been observed (Davis and Brown, 1927; Duke and Wallace, 1930; Brown, 1933; French, 1937; Gall et al., 1957). Each of these phenomena has been
related to the adsorption of serum globulins or anti-
trypanosomal antibody plus complement onto blood cell sur-
faces; the globulin adsorption alters membrane net elec-
trical charge and thus causes cell clumping and increased
erthrocyte sedimentation as well as attachment of
trypanosomes (Wallace and Wormall, 1931; Raffel, 1934).
Several authors have suggested that the adsorption of
immunoglobulins onto erythrocytes may contribute to ery-
throphagocytosis resulting in the anemia of trypanosomiasis
(Zuckerman, 1964). In addition, immunoconglutinin and cold
agglutinins to erythrocytes arise in the sera of some
trypanosome-infected humans and animals (Stats and Wasser-
man, 1943; Ingram and Soltys, 1960). None of these
reactions has been implicated in the pathology of try-
panosomiasis, however.

Autoantibody to normal tissues has been found in
experimental infections with the Brucei group African
trypanosomes. Muschel et al. (1961) and Seed and Gam
(1967) have demonstrated that CF autoantibodies to liver
and other tissues are produced in rabbits infected with
the human trypanosomes (T. gambiense and T. rhodesiense).
No role could be established for the autoantibody since
passive transfer of serum was not attempted in these investigations, and the immunoglobulin class(es) involved were not elucidated. Recently, MacKenzie and Boreham (1971) have demonstrated the possible occurrence of precipitating antibody to rabbit liver in a preliminary report of autoantibody in experimental T. brucei infections.

There have been no reports of autoimmunity to normal tissues in natural or experimental infections with trypanosomes of the Vivax or Congolense groups, those trypanosomes that are serious pathogens of domestic animals. Results presented in this investigation, however, clearly demonstrate that autoantibodies to normal tissue antigens arise during chronic T. congolense infections of rabbit. Titers of autoantibody rose rapidly one week after inoculation to rather stable levels at about two or three weeks (Figure 9); after chemotherapy, autoantibody titers gradually fell to preinfection levels or disappeared. CF results were substantiated by gel diffusion tests which demonstrated precipitating activity of infected rabbit serum with tissue extracts (Figure 19).

The normal occurrence of low levels of autoantibody
in apparently healthy rabbits was first reported by Kidd and Friedewald (1942a and 1942b) and has been well-documented by Asherson and Dumonde (1963 and 1964), and others; the "normal" autoantibodies arise in young rabbits at about two months of age and persist in the adult animal. The autoantibodies are specific for extracts of several tissues and are thought to arise as the result of *Eimeria stiedae* infections common to most rabbits (Asherson and Rose, 1963). Results presented here do not determine whether increases in preexisting autoantibody occur or whether "new" autoantibody arises. The author suspects that *T. congolense*-induced autoantibody rises anew, directed to different determinants than "Kidd-Friedewald" antibody, since secondary responses of normal autoantibody induced with injections of rabbit tissue gave rise to a predominantly IgG response (Asherson and Dumonde, 1964); this contrasts with the type of autoantibody immunoglobulin class predominant during *T. congolense* infections.

Landsteiner and Van der Scheer (1927) demonstrated Wasserman antibody production in rabbits following injections of phenolized *T. equiperdum*; consequently,
V.D.R.L. tests were performed on rabbit sera used in this investigation for autoimmunity. Negative V.D.R.L. results show that Wasserman antibody was not produced by infection with *T. congoense* and that such antibody was not a complicating factor reacting with some lipoidal moiety of extracted tissues or, conversely, that the autoantigens of this study were not similar to cardiolipin. Similar results were obtained by Muschel *et al.* (1961) and Seed and Gam (1967) in *T. gambiense* and *T. rhodesiense*-infected rabbits.

The chemical nature of the different tissue antigens was not determined in this investigation. Rough correlations exist, however, between protein concentration and antigenic strength of the tissue extracts. For example, liver extract had the highest concentration of protein and also had the greatest antigenic strength; brain extract had the lowest protein concentration and the least antigenic strength (Tables 9 and 10). In addition, the optimal dilutions of tissue extracts for the CF tests were such that brain antigen contained the highest concentration of protein, liver the next highest and heart and kidney the lowest concentrations of protein; thus, this may account for greater serum CF activity with brain and liver and
lesser activity with heart and kidney. These observations suggest, albeit tentatively, that the autoimmune responses in *T. congoensis*-infected rabbits may be non-organ-specific responses directed against common protein or protein-associated antigens(s).

Rabbit IgM and IgG immunoglobulin classes of infected sera were separated on the basis of molecular size and weight, and the serum fractions containing these immunoglobulin classes were further characterized by electrophoretic mobility and antigenicity. When the various serum fractions were tested for CF activity with tissue antigens, all CF activity occurred in those fractions containing IgM (Figures 11 and 15). These findings indicate that the autoantibody produced to normal tissue antigens during infection is exclusively IgM. Further substantiation was obtained for this hypothesis by demonstrating that serum CF activity with tissue antigens was reduced by treatment with 0.2M 2-mercaptoethanol. The macromolecular nature of the antibody as well as a possibility of multiple antigenic specificities and the relative insensitivity of the precipitation test (Humphrey and White, 1970) may explain
the diffuse gel precipitation lines observed with tissues.

In the demonstrations of autoantibody in rabbits with Brucei group infections, no characterization of the immunoglobulin(s) involved was made (Muschel et al., 1961; Seed and Gam, 1967). MacKenzie and Boreham (1971) did show heat lability of the autoantibody in their investigation, however, and suggested that the autoantibody might be IgM.

IgM levels rise considerably in the serum of humans and domestic animals naturally infected with trypanosomes (Mattern, 1964; Cunningham et al., 1966; Rees, 1969). The elevation of IgM levels has not been well documented in experimentally-infected rabbits, although Mattern et al. (1963) detected such changes in T. equiperdum-infected rabbits and Seed et al. (1969) found that T. gambiense infections increased IgM concentrations in the serum of rabbits. Results presented here demonstrate that IgM levels also rise (up to four times normal levels) in T. congolense-infected rabbits. Although the IgM rise was shown definitively by radial immunodiffusion tests (Figure 17), the rise is also noticeable in serum gel filtration and ultracentrifugation profiles (Figures 10, 11, 14 and 15).
In man and other primates infected with Brucei trypanosomes, molecular IgM entities other than those directed against trypanosomes arise. These are thought to contribute to the elevated levels. Henderson-Begg (1946), Houba and Allison (1966) and Houba et al. (1969) have demonstrated that IgM immunoglobulins with specificities for sheep erythrocytes (non-sensitized sheep cell agglutinins) and for IgG molecules (rheumatoid factor-like M-antiglobulins) arise during infection; they subsequently showed that the M-antiglobulins bore no relationship to the progress of infection, that the sheep cell agglutinins could be absorbed from serum with trypanosome antigens, and that repeated absorptions of infected serum with trypanosomes and sheep cells failed to reduce IgM or IgG levels. Preliminary studies by the author revealed that sheep cell agglutinins do not arise in T. congolense-infected rabbits.

The IgM autoantibodies observed in this investigation may significantly contribute to enhanced IgM levels during T. congolense infections, although this remains to be tested by more effective procedures than those presented in this paper. One must be prepared to accept the possibility, however, that in certain animals induced to form
specific antibody, the total gamma-globulin increase may not be immunoglobulins specifically directed against the inducing antigen (Askonas and Humphrey, 1958; Neal et al., 1969; Freeman et al., 1970).

Several theories on the induction of autoimmunity during infectious disease have been formulated. One theory is that some microbial antigens are antigenically similar to host antigens, and that antibody produced to the microbial antigens cross-reacts with host antigens (Zuckerman, 1964; Dameshek, 1965; Adams, 1969; Asherson, 1971). In this investigation, the inability to remove serum CF activity for tissues by absorbing with trypanosome extract indicates that antityrpanosomal antibody cross-reactive with tissue antigens was not a factor influencing the rise of autoantibody during disease (Table 8). The same results were obtained by Seed and Gam (1967).

Another theory is one that pertains to parasites which are intracellular (e.g. viruses) or which have intracellular stages; the microorganisms acquire host antigens and influence host antibody-producing cells in a hapten-carrier effect to produce autoantibody (Lindenmann
and Klein, 1967; Allison et al., 1971). Although Soltys and Woo (1969) found intracellular forms of *T. brucei* and *T. congolense* in infected mice, this phenomenon has not been substantiated by others nor shown to occur in any of the chronic infections. Indeed, histological sections taken from infected rabbit tissues in this work, and similar evidence from the work of Losos and Ikede (1970) and Wosu (1971), do not reveal any intracellular forms of *T. congolense*.

Several investigators, including Boyden (1964), Dameshek (1965) and Adams (1969), have postulated that intracellular or "sequestered" autologous antigens exposed to the circulation following tissue trauma may give rise to autoantibodies. In light of the extensive tissue necrosis observed in chronic *T. congolense* infections, such a theory could account for the production of autoantibodies. One argument against this mechanism is that the autoantibodies arise well before any grossly observable tissue necrosis occurs in *T. congolense* infections.

Another possible mechanism that cannot be eliminated in this study is that direct microbial action or meta-
bolic products alter host tissues enough to cause auto-
antigenicity, and that the resultant autoantibody cross-
reacts with normal tissues in in vitro tests (Humphrey
and White, 1970; Asherson, 1971).

An interesting theory of thymic dependent (T) lymphoto-
cyte and bursal equivalent (B) lymphocyte homeostatic
control in relation to autoimmunity has been promoted by
Teague and Friou (1969) and Allison et al. (1971). They
have postulated, based on considerable experimental
evidence, that T-lymphocytes have active and passive
control over such B-lymphocyte functions as the production
of autoantibodies; under certain circumstances, including
infectious disease, T-lymphocyte control over B-cells may
be altered or broken resulting in autoantibody formation.
This hypothesis will be discussed below in relation to
observations on cell-mediated events in African trypano-
somiasis.

The symptoms and pathology observed in rabbits infec-
ted with T. congoense conform closely to those observed in
previous studies with the same strain of parasite (Mahmoud,
1970; Wosu, 1971). The slight decrease of hematocrit
values in infected rabbits may have been due to hemodilution
resulting from vascular impairment (Boreham and Goodwin, 1966), adhesion phenomenon-aggravated erythrophagocytosis (Zuckerman, 1964), or autoantibody to erythrocytes. However, preliminary studies by the author revealed no agglutinating autoantibody for normal allogeneic erythrocytes, autologous erythrocytes or trypsinized erythrocytes. Histological sections of infected rabbit organs revealed no pathology except for that of the kidney.

In this investigation, passive transfer of infected rabbit sera or sera mixed with tissue antigens failed to reproduce the disease or its signs and external pathology. Although these results may suggest that T. congolense-induced autoantibody does not have immunopathologic capacity, such an assumption may be false. For example, the autoantibodies may have "inapparent" pathologic functions such as the blocking of membrane sites that are physiologically or pharmacologically important recognition sites for enzymes or hormones (Lennon and Carnegie, 1971), cytotoxicity for certain immunologically important cells as lymphocytes or neutrophils (Walford et al., 1965), or the coating of certain tissues to prevent adequate nutrient and gas exchange with the blood.
Conversely, the autoantibody may actually be beneficial to the host. Paterson et al. (1965) and Paterson (1968) have reported that CF antibrain IgM autoantibody can protect rats against the cell-mediated effects of experimental allergic encephalomyelitis. Boyden (1964) suggests that some autoantibodies may be part of a physiological "clearing mechanism" for cellular debris released during tissue damage. In any event the role, if any, of autoantibody formed during chronic *T. congolense* infections of rabbit remains to be elucidated.

In vivo and in vitro tests were used in this work to examine for cell-mediated sensitivity (classical delayed-type hypersensitivity) to normal brain antigens as well as to trypanosome antigens. MIF and SRF tests are valid in vitro correlates of delayed hypersensitivity and they have been used extensively in tests for cell-mediated immunity to many antigens including brain and protozoan antigens (David et al., 1964a and 1964b; Bloom and Bennett, 1970; Tremonti and Walton, 1970; Blewett et al., 1971; Rocklin et al., 1971).

That cell-mediated responses to brain antigens do not occur during chronic *T. congolense* infections of
rabbit was shown by the negative skin test, MIF and SRF test results (Tables 11 and 12). Autoimmunity in these infections, then, is probably limited to autoantibody production against normal tissue antigens. This finding was substantiated by examination of histological sections of infected rabbit organs which revealed that there were no mononuclear cell infiltrates and by preliminary observations of negative skin tests with the other tissue extracts.

Although there are no reports in the literature of cell-mediated autoimmunity in African trypanosomiasis, there is histopathological evidence for it. Baldwin (1904), Janssen and Van Bogaret (1956), Manuelidas et al. (1965) and Koten and DeRaadt (1969) have reported encephalitis and mononuclear cell foci within tissues of man and animal infected with Brucei group trypanosomes. Comparable lesions have not been recorded in animals with Congolense group trypanosome infections, and this may be partially due to an absence of central nervous system involvement of animals infected with the parasites.

Although the literature abounds with reports of cell-mediated hypersensitivity to microbial antigens (reviewed
by: Samter, 1965; Gell and Coombs, 1968; and, Miescher and Muller-Eberhard, 1968), there have been no reports of such phenomena occurring or not occurring in African trypanosomiasis. The present investigation revealed no cell-mediated responses to trypanosome extracts prepared from the infecting *T. congolense* strain and from substrains derived from infected rabbits at biweekly intervals (Tables 11 and 12). In the typically chronic trypanosome infections of rabbits, the parasites are present in very low numbers and may be concentrated in peripheral blood vessels (Seed and Gam, 1967); in such a situation it could be advantageous for the host if cell-mediated responses to parasite antigens could be mounted. Future investigations of the cellular immunoresponsiveness of trypanosome-infected animals should include attempts to specifically sensitize the animals to common or non-variant antigens so that cell-mediated responses prevail. There is evidence that cell-mediated immunity may be more effective in subduing antigenically-variant parasite populations than humoral immunity (Brown, 1971) and, indeed, may be more effective in localizing parasites within the tissues or at the site of insect inoculation (Bryceson, 1970).
Part of the pathogenesis of trypanosomiasis may be due to parasite effects, direct or indirect, upon the T-lymphocyte. Allt et al. (1971) have recently demonstrated that the cell-mediated effects of experimental allergic neuritis in rabbits may be suppressed by trypanosome infection. Goodwin (1970) relates that humoral anti-SRBC responses in mice and rabbits, in which such responses are known to require T-lymphocyte cooperation, can be suppressed by trypanosome infection. Ormerod (1961) notes that there is a high incidence of neoplasia occurring in the terminal stages of human trypanosomiasis, which would indicate a breakdown of the immunological surveillance system. In addition, autoantibody production in infectious disease has been correlated with T-lymphocyte malfunction (Allison et al., 1971).

In observing the theories of Teague and Friou (1969) and Allison et al. (1971) on the importance of T-lymphocyte responsiveness in immunologic homeostasis, it is tempting to formulate an hypothesis that chronic trypanosome infections somehow affect the regulatory and control functions of T-lymphocytes and that autoantibody production, the lack or suppression of cell-mediated responses, and the
lack of cooperation with B-lymphocytes in producing antibody to certain antigens are all indicators of T-lymphocyte malfunction. This hypothesis remains to be tested.

Additional evidence for trypanosome-induced immunopathology is revealed in this investigation. Skin tests with trypanosome extracts in infected and recovered rabbits, which had circulating precipitating antibody to parasite antigens (Figure 18), produced an immediate hypersensitivity response; the response was probably directed to parasite antigens of $MW > 5 \times 10^4$. The temporal sequence of events in these skin responses (initiation, duration and intensity) was suggestive of Arthus reactions (Miescher and Muller-Eberhard, 1968; Humphrey and White, 1970). Substantiating evidence was found in histological analyses of the skin reactions (Figures 21 to 26); the histology matched the classical cytological picture of Arthus reactions as described by Martins and Raffel (1964), and was distinct from that of delayed hypersensitivity and "Jones-Mote" reactions. Further evidence for the Arthus-type reactions was obtained by passive transfer of lesions to normal rabbits with infected rabbit serum-trypanosome extract immune complexes.
The direct demonstration of antigen-antibody mediated immediate hypersensitivity in animals with African trypanosomiasis has not been previously reported. However, such reactions have been suggested as the root cause of the increased kinin release and vascular disruption observed in Brucei group trypanosome infections of rabbit (Boreham and Goodwin, 1966; Boreham, 1968a and 1968b; Goodwin and Hook, 1969; Goodwin, 1970). Seed (1969) injected viable T. gambiense intradermally into normal rabbits and observed skin reactions that developed at the site of inoculation one or two weeks afterwards; although Seed interpreted the skin reactions as demonstrating trypanosomal toxin, the reactions may have been caused by antibody combining with parasite antigens at the site of inoculation.

Indeed, an interesting hypothesis wherein trypanosome antigen-antibody complexes cause external and internal pathology may be formulated, with ongoing immediate hypersensitivity reactions of the Arthus type, both local and systemic, ultimately responsible for events leading to pathology and death in T. congolense-infected animals.

Parasite antigen-antibody reactions in blood vessels with the subsequent fixation of complement could summon
forth a PMN response; the PMN's, after phagocytizing the immune complexes, could lyse and release lytic enzymes into the tissues causing necrosis, the release of pharmacologically active agents and vascular disruption. Sustained vascular disruption may lead to death. The sites of immune complex localization may vary with the species of animal that is infected, but in rabbits they are probably areas of skin, genitals and the kidneys.

Supportive evidence for the hypothesis that the pathology of *T. congolense* infections is a result of immediate hypersensitivity is found in the descriptions of rising titers of antibody to parasites, the subsequent disappearance of parasites and the onset of symptoms and pathology during *T. congolense* infection of rabbits and cattle (Fiennes, 1950; Wosu, 1971). Further evidence is obtained by comparing histologic sections from necrotic skin and organs of infected rabbits (Wosu, 1971) with sections from the Arthus lesions and infected organs of rabbits studied in this investigation. Both show PMN perivascular cuffing and accumulation within the skin, as well as necrosis, and reveal glomerular disruption in the kidney. Also, Boreham and Kimber (1970) describe detection by fluorescent antibody techniques of immune
complexes in *T. brucei*-infected rabbit kidney arterioles and venules but not in other organs. Proper testing of this hypothesis will involve the monitoring of complement levels during disease, the detection of immune complexes in the vessel walls of disrupted tissues and observations on the course of disease in complement-depleted or leucocyte-depleted animals.

One application of the skin test reactions observed in infected rabbits would be as a diagnostic skin test for trypanosomiasis. The trypanosome extracts prepared for this investigation contain primarily non-variant internal antigens in addition to external antigens (Mahmoud, 1970), and would theoretically be capable of eliciting a skin reaction at any stage of disease if antibody is present. Evidence for this possibility comes from observations by Roberts *et al.* (1969) on local skin reactions in *T. congolense* infected cattle that occur at the site of tsetse inoculation. These skin reactions appeared seven to eleven days after the tsetse bite (long enough for antibody production), and trypanosomes were found within the skin reaction nodules until the parasites were observed in the blood; the reactions then gradually disappeared.
An overview of this investigation reveals that potentially immunopathologic mechanisms occur in T. congolense-infected rabbits. Autoantibody arises during infection and reacts in CF and gel diffusion tests with normal tissue antigens. No cell-mediated immunity to tissue antigens or to parasite antigens was found, but Arthus type immediate hypersensitivity to trypanosome antigens was demonstrated. A role for autoantibody remains to be elucidated. The author suggests that local and systemic Arthus reactions may be responsible for much of the pathology observed in infected animals, and that impairment of T-lymphocyte function may also occur during trypanosomiasis.
SUMMARY

1. Potentially immunopathologic mechanisms have been investigated in rabbits with experimental *Trypanosoma congolense* infections.

2. Complement fixing and precipitating autoantibodies to normal rabbit tissues arise during infection. The CF activity of infected rabbit serum was graded as brain > liver > heart > kidney; the graded response to the different tissues may have been due to the differences in protein concentration of the tissues at their optimal dilution for CF test.

3. The occurrence of "normal" autoantibody to rabbit tissues was reconfirmed. Data presented in this study do not determine whether autoantibody was formed anew or occurred as a rise in preexisting autoantibody.

4. The autoantibody was of the IgM class as shown by several physico-chemical methods of analysis; IgM autoantibody may have contributed to the elevated IgM levels observed in infected rabbits.
5. Passive transfer of pooled infected rabbit serum to normal rabbits failed to reproduce the disease or its external symptoms. The role of *T. congolense*-induced autoantibody remains to be elucidated.

6. Wasserman antibody did not arise in infected rabbits.

7. Cell-mediated autoimmunity to normal tissues did not occur during infections, as shown by histological sections of infected rabbit organs, skin tests, MIF and SRF tests.

8. Macroscopic histopathology was confined to the face, ears and genitals of infected rabbits. Enlarged spleens were noted. Microscopic degeneration of kidney glomeruli and tubules was observed, but all other infected rabbit organs appeared as normal.

9. Immediate hypersensitivity of the Arthus type was demonstrated in the skin of infected and recovered rabbits given intradermal injections of trypanosome extracts; histologic sections revealed that approximately 95% of all infiltrating cells were PMN's at 24 hr after injection. The author suggests that on-going local and systemic immediate hypersensitivity reactions as a result of trypanosome antigen-antibody
immune complexes being deposited in blood vessels or tissues may be responsible for pathology and death in \textit{T. congolense} infections.

10. The Arthus lesions could be passively transferred to normal rabbit skins with mixtures of antiserum and trypanosome extract in concentrations of antigen excess for a precipitation reaction.

11. Skin test diagnosis of \textit{T. congolense}-infected animals may be possible with trypanosome extracts containing, predominantly, the non-variant trypanosome antigens.

12. Cell-mediated hypersensitivity to trypanosome extracts was not detected in histological sections, by skin tests, MIF and SRF tests.

13. The author suggests that chronic trypanosome infections of the type induced in rabbits with \textit{T. congolense} may alter T-lymphocyte function, and that autoantibody production and the absence of cell-mediated events during infection are indicators of this malfunction.
APPENDIX

Sources of materials and reagents used in this study.

Amicon, Inc., Lexington, Massachusetts
A.O. Spencer Co., Buffalo, New York
Beckman Instruments, Inc., Palo Alto, California
Boots Pure Drug Co., Ltd., Nottingham, England
Calbiochem, San Diego, California
Chromatography Corporation of America (out of business)
CIBA Pharmaceutical Co., Summit, New Jersey
Consolidated Laboratories, Inc., Chicago Hts., Illinois
Cordis Laboratories, Inc., Miami, Florida
Eli Lilly and Co., Indianapolis, Indiana
Falcon Plastics, Oxnard, California
General Biochemicals, Chagrin Falls, Ohio
Grand Island Biological Co., Grand Island, New York
Humble Oil and Refining Co., Linden, New Jersey
ISCO, Lincoln, Nebraska
Kontes Glass Co., Vineland, New Jersey
Lederle Laboratories, Pearl River, New York
LKB-Produkter AB, Stockholm 12, Sweden
Miles Laboratories, Inc., Kankakee, Illinois
Millipore Corporation, Bedford, Massachusetts
Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey
Republic Seitz Filter Corporation, Milldale, Connecticut
W. and R. Balston, Ltd., Maidstone, England
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