Anti-Immunoglobulins and Heterophil Agglutinins in Experimental Trypanosomiasis

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Rheumatoid factor (RF)-like substances and heterophil agglutinins (HA) were induced in rabbits by infection with a strain of *Trypanosoma equiperdum*. Infection with a *T. gambiense* strain produced HA, but no RF. RFs appeared usually before immunoglobulin G (IgG) levels started to increase. They disappeared spontaneously after 4 to 6 weeks. HA were of the IgM class, appeared within 1 to 2 weeks after infection, and usually remained at high levels afterwards. They could not be absorbed out with lyophilized whole trypanosomes. Therapy suppressed the formation of RF as well as of HA, but IgG complexes as well as antibodies against trypanosomal antigens remained present for considerable periods after the installment of therapy. It is concluded that RFs are not induced by IgG complexes. The results suggest that HA are probably not cross-reacting antibodies induced by trypanosomal antigens.

In 1970 Klein et al. (9) demonstrated the induction of anti-immunoglobulins (rheumatoid factor [RF]-like substances) in rabbits infected with *Trypanosoma equiperdum*. These antibodies were immunoglobulin M (IgM), directed against heterologous (human) IgG. The authors suggested that this phenomenon could provide a model for investigating the nature of anti-immunoglobulin formation. This has been attempted in the present study, which also includes a confirmation and extension of our previous work. This study is a longitudinal one in which several animals and different parameters have been followed for prolonged periods of time. In particular, the relation of anti-immunoglobulins to their supposed antigens, i.e., IgG immune complexes, has been investigated.

Attention was focused on those anti-immunoglobulins that are considered most relevant for rheumatoid arthritis (RA), i.e., those detected in the latex fixation and Waaler-Rose tests (18).

Observations on the formation of heterophil agglutinins (HA) and on their possible antigens are reported as well. The influence of therapy on these phenomena has been studied.

MATERIALS AND METHODS

Experimental animals and parasites. Outbred rabbits of the wild type were obtained commercially, always from the same firm. The infecting organisms were the following. *T. equiperdum* Pasteur Paris was obtained from Colas Belcour in 1961, in some instances inoculated as clone BoTat-1 (Eq.I.P.). From this strain, 101 clones (antigenic types) have been isolated (1). Of these, the clone BoTat-1 is hyperdominant.

This implies that without previous cloning BoTat-1 will always be the second antigenic type to appear after infection. The strain T.eq. Pasteur Paris characteristically produces an acute infection resulting in lethality within 4 to 6 weeks. The infection is accompanied by a considerable increase in serum IgM level in rabbits (9). *T. gambiense* D2, clone BoTat-134, was isolated by P. Mattern in 1963 at Rufisque (Senegal) from the cervical lymph node of a patient in the nervous stage of sleeping sickness. This strain characteristically tends to produce a chronic infection (lasting up to 6 months) in mice.

Animals were infected intraperitoneally with amounts of *T. equiperdum* varying from 2×10^4 to 5×10^7 or amounts of *T. gambiense* varying from 10^3 to 10^6 without any apparent difference in results.

Lyophilisates of isolated trypanosomes (T.eq. Pasteur Paris BoTat-1) were prepared by passing infected rat blood through diethylaminoethyl-cellulose as described by Lanham and Godfrey (10). The resulting suspension was centrifuged, washed with phosphate-buffered saline containing 0.9% glucose, dialyzed against phosphate-buffered saline without glucose, centrifuged again, and freeze dried.

Therapy. In most cases Arsobal (=melarsoprol, melaminyl-4-phenyl-arsenosodithioglycerine, Specia, Paris) was used. Once a day, 0.1 ml of a 3.6% solution was injected intramuscularly for 5 days. Four hours before and after each injection, 1 ml of 2.5% Phenergan solution was given intramuscularly to avoid anaphylactic shock. In a few cases the irradiation treatment described by Pautrizel et al. (16) was used. Only those cases were considered in which therapy was successful. Successful therapy was defined as the lasting disappearance of clinical symptoms as well as negative parasitemia in mice after injection of 0.5 ml of the blood under investigation.

Blood samples were taken weekly, and the sera were analyzed for various components.

Specific antisera against rabbit IgG (in goat or swine) and IgM (in sheep) were obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands).

Quantitative analysis of IgG. The radial immunodiffusion test gave inaccurate results for the quantitation of IgM and IgG in these sera because calibration lines obtained from preparations from different sera did not always coincide within the experimental error. Such deviations were less apparent in the simpler double-diffusion method in serial dilution. By this method approximate immunoglobulin levels are obtained relative to a serum sample taken before the infection (13).

To match concentrations of immunoglobulins of different sera in zone centrifugation experiments (see Detection of IgG complexes in rabbit sera), we used electrophoretic scanning. Sera were separated on Cellogel cellulose acetate strips and stained with Ponceau red, and the gamma fraction was calculated as the percentage of total protein as determined by a biuret method. This method gave an acceptable correlation with immunological IgG determinations in those cases where valid calibration lines were obtained.

Serological tests. The latex fixation test (19) was slightly modified as follows. All sera were inactivated previously for 10 min at 56°C. The mixture of diluted serum samples with coated latex was incubated for 30 min instead of the usual 90 min. The first serum dilution was 1/10 before the addition of latex suspension. Clq is not detected by this procedure. The human erythrocyte agglutination test (19) was a modification of the Waaler-Rose test with human O Rh plus erythrocytes sensitized with rabbit antiserum.

HA were detected by adding a 0.3% tanned and fixed sheep cell suspension in phosphate-buffered saline (15) (kindly supplied by A. Leeuwenberg) to a serial dilution of from 1/4 to 1/1,024 of serum in phosphate-buffered saline.

For absorption of HA, quantities ranging from 5 to 20 mg of lyophilized trypanosomes were gently stirred with 0.5 ml of serum for 3 h at 37°C and then kept overnight at 4°C. After centrifuging HA titers were determined and compared with those of an untreated serum sample.

Antibodies against trypanosomal antigens. Hemagglutinating antibodies were detected by a system containing extracted trypanosomal antigens fixed by glutaraldehyde to sheep erythrocytes as described by Capbern et al. (2).

Detection of IgG complexes in rabbit sera. IgG complexes were detected by zonal centrifugation of whole serum in a sucrose gradient made of three equal layers of 15, 25, and 40% sucrose in phosphate-buffered saline (wt/vol). To each 5-ml tube, 0.1 ml of serum in ca. twofold dilution was applied. The dilutions of sera to be compared in one run were chosen to match the immunoglobulin fractions as determined by electrophoretic scanning. The centrifugation was performed in the swing-out rotor (3 by 5 ml) of an MSE 65 Superspeed ultracentrifuge for 17 h at an average of $100,000 \times g$ (34,000 rpm) and at room temperature.

Fractions of about 0.5 ml each were collected by piercing the bottom of the tube with a hollow needle. The sedimentation front of the IgG zone in the tube

was localized by double diffusion of the fractions against specific anti-IgG antiserum. In the figures, IgG complexes (bars) are expressed as the percent difference in sedimentation with a standard consisting of a preinfection serum from the same animal. Differences of more than one tube fraction between sedimentation fronts in the same run were considered significant and were interpreted as having been caused by IgG heavier than the native monomer. In a series of measurements in the same animal, IgG complexes were considered to be present if a difference of two fractions between serum and reference was accompanied by at least one difference of one fraction or more (at least 5% more sedimentation) for another serum in the same series.

It has been shown that without added albumin, immune complexes dissociate easily on gel filtration (7). This can also happen in the zone centrifugation procedure, but to a lesser extent. Our centrifugation experiments, which were carried out before these results became available, may therefore underestimate the amount of IgG complexes.

RESULTS

Formation of anti-immunoglobulins in infected animals without therapy. Thirteen rabbits were infected with *T. equiperdum* without any therapy. Figure 1 gives a representative example. In this figure the course of total IgM and IgG levels, latex fixation titers, and immune complexes is given as a function of time. Similar results were obtained in the other cases. Table 1 summarizes these results.

Common traits in these longitudinal observation series of anti-immunoglobulin formation were: (i) early appearance of anti-immunoglobulins, in most cases 1 to 2 weeks after the infection; (ii) disappearance of positive latex titers after 4 to 6 weeks in those cases where the observation time was long enough; (iii) in the five cases in which IgM was estimated, the latex titer and the IgM level increased in a roughly parallel way. In the later stages this parallelism disappeared. The increase in IgG levels nearly always started somewhat later than either the IgM increase or anti-immunoglobulin development.

Eight rabbits infected with *T. gambiense* (clone BoTat-134) remained negative or developed only weak or irregular anti-immunoglobulin titers in the latex test.

Anti-immunoglobulins in infected animals given therapy. Eight animals were infected with *T. equiperdum*. After various time intervals, antitrypanosome therapy was begun. The effect on anti-immunoglobulin formation (latex test) is illustrated in Fig. 2. Early therapy (within 1 week) suppressed the formation of anti-immunoglobulins (Table 2). In Fig. 3 a case is shown (RR13) in which two subsequent infections with simultaneous therapy led to the com-

FIG. 3. Parameters related to infection with *T. equiperdum* in a treated rabbit (case RR 13). Symbols: $\square$, Passive hemagglutination (H); $\blacksquare$, HA; other symbols as in Fig. 1.

anti-immunoglobulins as revealed by the latex test developed over this period of time, except in trace amounts.

Results of Waaler-Rose tests. In 24 animals, with and without therapy, Waaler-Rose titers were determined. Their course was quite irregular, and their presence or absence had no relation with any parameter of the infection. Moreover, when positive results were obtained, similar titers were often found with unsensitized erythrocytes. For these reasons the results of Waaler-Rose tests are thought not to be indicative of anti-immunoglobulin formation resulting from the infection.

HA. In four untreated rabbits infected with *T. equiperdum* and in four untreated rabbits infected with *T. gambiense*, agglutinin titers were followed. Figure 4 gives a representative example. In the *T. equiperdum* cases, agglutinin titers appeared, usually after 1 or 2 weeks, and reached a maximum after 3 to 4 weeks (Table 3). From the four *T. gambiense* cases, two behaved in the same way: one produced hardly any agglutinin, and one had a high titer before infection. Once they had appeared, titers usually stayed during the period of observation, contrary to the behavior of the anti-immunoglobulins. Gel filtration of six sera through Sephadex G-200 gave agglutination only in fractions containing IgM.

Antitrypanosome therapy suppressed the formation of HA antibodies in seven cases (Table 4). It can be seen that irradiation therapy does

FIG. 4. HA and antibodies against trypanosomal antigens (H) in two rabbits infected with *T. equiperdum*. HA in case RR 20 (treated) ($\circ$) and in case B 11 (untreated) (Δ); H in case RR 20 (treated) ($\bullet$) and in case B 11 (untreated) ($\blacktriangle$).

not always completely suppress HA formation. Case RR 13 (Fig. 3) formed little HA after therapy, but produced considerable titers after reinfection without therapy, just as was observed with the anti-immunoglobulins.

Absorption experiments were carried out with

TABLE 3. HA titers in rabbits infected with *T. equiperdum* or *T. gambiense* and their time course in weeks after infection

Rabbit no.	Appearance after week:	Maximum titer after week:	Maximum titer
B 9	1	3	≥2,048
B 11	1	4	≥2,048
B 12	1	4	512
RR 13	3	7	≥2,048
A 10 ^a	0	6	8 (background)
A 11 ^a	3	4	256
A 14 ^a	1	2	256

^a *T. gambiense*.TABLE 4. Time course in weeks of HA titers in rabbits infected with *T. equiperdum* and treated therapeutically

Rabbit no.	Therapy started after week:	Maximum titer after week:	Disappearance of HA after week:
B 5 ^a	1	2	3
B 7 ^a	4	4	5
RR 18 ^a	1	1	2
RR 19 ^a	1	1	5
RR 20 ^a	1	2	6
RR 13 ^b	Simultaneously	4 (titer 16)	5
RR 46 ^b	Simultaneously	No titer	

^a Chemotherapy.^b Irradiation therapy.

considerable amounts of (10 to 40 mg/ml of serum) of homologous lyophilized trypanosomes, but no measurable amounts of HA were absorbed from six sera of different animals.

The course of HA titers was different from that of hemagglutinating antibodies against trypanosomal antigens. In all treated animals, these antibodies continued to increase long after the HA had disappeared (Fig. 4).

DISCUSSION

This study confirms previous work (9) with different types of rabbits and under different conditions. Because a *T. gambiense* strain gave negative results with respect to RF formation, it is possible that the induction of RF-like substances is strain dependent. Such phenomena might account for the negative results reported by Ross and van Regenmortel (17) in a comparable model. However, since the rabbits used were outbred, genetic influences of the host cannot be excluded either. The preference for a heterologous IgG substrate was found in humans infected with *T. gambiense* (8). The disappear-

ance of anti-immunoglobulins soon after the initiation of therapy and in some cases their complete suppression by early therapy prove their close relationship with the infection.

Current hypotheses about the origin of RF suppose that immune complexes of autologous IgG are the primary antigen of RF. In subacute bacterial endocarditis RF-like substances directed against isologous and heterologous IgG are formed which disappear after antibacterial therapy (20). Also, there is a parallelism between RF titer and IgG level (14). These phenomena were reproduced in rabbits immunized with killed staphylococci from such infections (21). Such experiments suggest that autologous IgG complexes are in fact the antigens of rheumatoid factors. This suggestion has been confirmed recently (3) when a relationship in time was found between RF and immune complexes as in human subacute bacterial endocarditis, and a suppressing influence of therapy on both variables. If this were true for our model, a relationship between IgG complex circulation and anti-immunoglobulin formation could also be expected. In our experiments, however, no such relation has been found because IgG complexes were present also in cases where anti-immunoglobulin formation had been suppressed by antitrypanosomal therapy. Furthermore, the expected parallel between anti-immunoglobulin titer and the increase of IgG level was absent in our experiments and anti-immunoglobulins appeared much earlier than in the experiments with killed staphylococci (21). The present model differs from most experimental RF models in that live organisms were introduced instead of killed ones. According to Greenwood's hypothesis (4), nonspecific B-cell mitogens might be produced by the infecting trypanosome. These mitogens might stimulate B-cells to produce various antibodies not necessarily directed against antigen formed in the disease process. These could be RF-like substances, HA, or antinuclear factors (11). Evidence for a trypanosomal mitogen in rabbits was obtained by Mansfield et al. (12). These authors suggest that production of nonspecific antibodies could take place when such a mitogen acts on B-cells while at the same time suppressor T-cells are blocked.

Other antibodies with similar origin might be the HA which have been found in human trypanosomiasis (5). These antibodies have been induced experimentally in monkeys infected by *T. gambiense* (6). An origin as a cross-reacting antibody against the trypanosome was suggested by absorption experiments (6). In our experiments the HA behaved like the anti-immunoglobulins with regard to early appearance, influence of therapy, and lack of relationship to the

supposed antigen. Also, after therapy, the HA behaved unlike antitrypanosomal antibodies as detected by passive hemagglutination. Although we could not absorb out the HA with whole trypanosomes, it might still be possible that some treatment would unmask a cross-reacting antigen or that an exo-antigen of the trypanosome might have produced these antibodies.

The present results would appear to point to a non-antigen-specific origin of RF-like substances and perhaps also of HA as the result of an external action (i.e., an invading organism) on an otherwise normal immune system. The extrapolation of such conclusions to the formation of RF in RA is another step that cannot yet be taken but that has become conceivable by these experiments. The discrepancy with the SBE model would suggest that there may exist more than one mechanism of RF formation.

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