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OBSERVATIONS ON THE ANTIGENS OF SOME TRYPANOSOMES WITH SPECIAL REFERENCE TO COMMON ANTIGENS

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Subsequent to the pioneering investigations earlier in the century (reviewed by Taliaferro, 1930) immunologists resumed the intensive study of relapses caused by African trypanosomes.

Trypanosomes of the *brucei* group have recently been shown to contain a high concentration of soluble specific antigens by Weitz (1960), Brown & Williamson (1962, 1964), Williamson & Brown (1964) and Seed (1963). These elicit a specific antibody response demonstrable by neutralization (Soltys, 1957), agglutination (Gray, 1962; Brown & Williamson, 1964) and precipitin tests (Brown & Williamson, 1962, 1964; Seed, 1963; Williamson & Brown, 1964). The appearance of antibodies is followed by a temporary disappearance of parasites from the blood stream. Soon, however, a new population of trypanosomes appears, shown by *in vitro* tests to be resistant to the agglutinating, trypanocidal and neutralizing antibodies directed against its predecessors. Such populations have been called antibody-resistant, relapse, or variant strains.

Although these variable, specific antigens obviously have specific antigenic determinants, biophysical and biochemical characterization indicates that basically their structures must be very similar (Brown & Williamson, 1962, 1964; Williamson & Brown, 1964).

Immunodiffusion tests (Brown & Williamson, 1962, 1964; Seed, 1963; Williamson & Brown, 1964) have shown that variant strains of a single species have antigens in common. Gray (1961) also found that *T. vivax*, *T. brucei* and *T. gambiense* share certain precipitinogens.

The object of this study was to compare the antigenic structure of a series of laboratory strains of trypanosomes by means of mainly immunodiffusion techniques. An antiserum directed against a single strain was used in most instances. All precipitinogens detected in heterologous strains by this antiserum could, therefore, be regarded as common to those of the homologous strain.

Materials and Methods

Trypanosome strains

Unless otherwise stated these were passaged in mice at three-day intervals. *T. rhodesiense* parent strain (P) was isolated from man in 1923. *T. rhodesiense* relapse strain (R) was obtained from the parent strain in 1960 by subcurative treatment of infected rats with trivalent tryparsamide (Williamson & Brown, 1964). After a few passages in rats and mice it was stored frozen at —80° C. *T. evansi* normal strain (N) was isolated from a camel in the Sudan in 1938, and the akinetoplastic strain of *T. evansi* (AK) was isolated from a camel in 1937. *T. equinum* (E)

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was obtained from the Argentine in 1948. The exact history of the *T. equiperdum* strain (EP) is unknown (Fulton & Spooner, 1959). *T. lewisi* (L) was isolated from a wild rat in England (Ryley, 1951) and passaged in laboratory rats every two weeks.

Preparation of antigens for immunodiffusion studies

" Homogenates " were prepared by separation of trypanosomes from heavily infected rat blood by differential centrifugation and disruption in a Mickle disintegrator as described by Williamson & Brown (1964).

The homogenate was centrifuged at 450g for 30 minutes at 4° C to remove larger particles, and the opalescent supernatant used as " crude antigen ". " Cell sap " of *T. rhodesiense* (P) was obtained by centrifugation of the latter at 150,000g for 60 minutes at 0° C. The protein concentration of these antigens was measured by the biuret reagent.

Pooled sera and a lyzate of blood cellular elements obtained from uninfected rats were used as control antigens. All antigens were stored at —20° C.

Antisera

Antisera to the parent (15/5 & 15/6) and relapse (16/2) strains of *T. rhodesiense* had been prepared in rabbits by Williamson & Brown (1964) and were stored frozen at —20° C.

Double diffusion precipitation tests according to the method of Ouchterlony (1949)

These were done in petri dishes containing 0·8 per cent Oxoid Ionagar in 0·067 M phosphate buffer (pH 7·7) with 0·05 per cent sodium azide as preservative. Wells 6 mm in diameter were cut with a cork borer after the required pattern and loaded with 0·04 ml of the reactants. The antigens were diluted to contain ±10 mg protein per ml. The dishes were held at 3° C for seven days and the precipitin lines which developed were recorded photographically.

Immunoelectrophoresis

A modification of the micro-immunoelectrophoresis technique of Scheidegger (1955) was used. Microscope slides (7·5 × 2·5 cm) or photographic glass plates (8 × 5·5 cm) were covered with an approximately 1 mm thick layer of 0·8 per cent Oxoid Ionagar in 0·067 M phosphate buffer (pH 8·0) containing preservative as above. Improvised stainless steel punches were used to cut wells 1·5 or 3·0 mm in diameter. Troughs 1 mm in width were cut with a scalpel.

Electrophoresis was carried out for 30 minutes with an applied potential of 160 volts. Petroleum ether of low boiling point (60 to 80° C) evaporated by a fan was used as coolant, as described by Wieme (1959). Precipitin lines were allowed to develop at room temperature by placing the slides in a moist chamber and recorded photographically. Permanent preparations were made by washing the slides in 0·9 per cent aqueous NaCl for 48 hours, drying the agar at 37° C and staining the lines with Naphthalene Black 10B.

Starch gel electrophoresis according to the method of Smithies (1955)

A discontinuous glycine-sodium hydroxide-borate buffer system at pH 8·6 (Fahey & Askonas, 1962) was used. Electrophoretic separation was carried out at a potential gradient of 8·3 volts/cm for 4½ hours at room temperature. The starch gel was sliced and stained with Amido Black 10B.

Agglutination tests

These were done according to the method described by Cunningham & Vickerman (1962). Blood obtained from mice showing a severe parasitaemia was stored at —80° C and thawed for use when necessary.

RESULTS

Double diffusion precipitation tests

Two precipitin lines due to antibodies against contaminating host antigens are visible in Fig. 1 and 2 opposite the well containing normal rat serum. They show reactions of identity with very faint lines opposite the well containing a lyzate of rat blood cellular elements (Fig. 2). A single line continues and is just visible opposite the *T. rhodesiense* (R) well; it disappears opposite the *T. equinum* well with its broad intense bands, which, like those of *T. rhodesiense* (P), tend to obscure the less distinct lines, making it impossible to determine their exact number.

Fig. 1 also shows that the *T. equinum* " homogenate " contains at least three precipitinogens. The lines cross those of the control serum well indicating that they are of parasite origin. The same applies to *T. equiperdum*. The bands of both *T. equinum* and *T. equiperdum* close to the central well appear to link up with those of *T. rhodesiense* (P), i.e. they are thus likely to be common with those of the latter. All three " homogenates " also have a slow moving antigen of high molecular weight as judged by the formation of a strongly curved line close to the antigen well (Crowle, 1961). Although these are not concurrent with each other, the similar patterns suggest that they are common precipitinogens. They do not appear to be present in " crude antigen " and " cell sap " (Fig. 2).

T. equinum " homogenate " contains at least four precipitinogens (Fig. 3). At least two of these show reactions of identity with those of the akinetoplastic strain of *T. evansi* (AK). These in turn link up with two lines of the normal strain of *T. evansi* (EV) which are almost superimposed. Both *T. equinum* and *T. evansi* (EV) have the slow-moving, macromolecular antigenic component referred to previously. It is also present in *T. rhodesiense* (R), but absent in *T. lewisi* " homogenate " (Fig. 4 & 5) and in the akinetoplastic strain of *T. evansi* (Fig. 3). Both *T. rhodesiense* (R) and *T. lewisi* have at least three antigens in common with *T. rhodesiense* (P).

Immunoelectrophoresis

No precipitin lines due to rat serum antigens and antibodies directed against them could be identified (Fig. 6). This means that the bands obtained by this technique can all be regarded as indicative of the presence of parasite precipitinogens. The reason for the apparent absence of serum antigens in this study probably lies in the use of smaller volumes of reagents. Fig. 6 also shows that " cell sap " of *T. rhodesiense* (P) contains at least four antigens. In other runs up to six were identified in " crude antigen " of this strain.

A simultaneous comparison of " crude antigens " of *T. rhodesiense* (P), *T. rhodesiense* (R) and *T. equinum*, using equal protein concentrations, is illustrated in Fig. 7. A similar run for *T. lewisi* has also been inserted. With homologous (15/6) serum the parent strain shows three lines. The one closest to the cathode is broad and distinct, and represents a specific antigen as it is absent in the relapse strain. With heterologous (16/2 = Anti-relapse) serum three lines are visible in the parent antigen. Their positions correspond to two of the three shown by the relapse antigen with the same (in this case homologous) serum. The third line of the latter antigen is also specific and corresponds to the specific line of the parent antigen in position and appearance.

A mixture of 15/6 and 15/5 serum (both anti-parent) reveals two distinct bands on the anodic and two very faint lines on the cathodic side of the origin in *T. equinum* and *T. rhodesiense* (R). The two faint lines of the latter were just discernible when 15/6 serum only was used. *T. lewisi* shows three bands of which one is much clearer than the others and corresponds in position to the electrophoretically faster moving precipitinogens of the other three trypanosome strains.

Starch gel electrophoresis

The electrophoretic patterns of " crude antigens " of *T. rhodesiense* and *T. equinum* as illustrated in Fig. 8 are so similar that differentiation on that basis is impossible. *T. lewisi*, however, is appreciably different, thus providing evidence against the possibility that the marked similarity seen in the two representatives of *brucei-evansi* trypanosomes is due to contaminating host antigens.

T. lewisi differs from them by the absence of visible zones in the region close to the cathode where the specific 4*S* antigens described by Williamson & Brown (1964) occur. *T. rhodesiense* (P) has at least four and *T. equinum* three zones in this region, of which two are quite concentrated. The most intense *T. lewisi* zones are crowded towards the anodic side of the run.

Agglutination tests

Homologous antiserum agglutinated *T. rhodesiense* (P) organisms up to a dilution of 1/80, whereas heterologous anti-*T. rhodesiense* (R) serum had no effect on these organisms.

Discussion

It is difficult to avoid confusing antigens of parasite and host origin, particularly when common antigens are being studied. Although the immunizing antigens used to produce antisera in this study were fairly pure, precipitating antibodies to host antigens were detected in low concentrations by including suitable controls.

In double diffusion precipitation tests with the Ouchterlony format (Ouchterlony, 1949), common antigens were identified by reactions of identity. The many bands present, however, tended to overlap and obscure the picture. Immunoelectrophoresis was therefore used in order to separate the different precipitinogens.

If physical conditions are kept fairly constant it is possible to identify common precipitinogens with fair accuracy by their electrophoretic mobilities in a comparative run, particularly if a single antiserum is used. Since, however, some of the precipitin bands differ in intensity and length, the issue may sometimes be confused. Nevertheless there can be no doubt that all the precipitinogens detected in heterologous antigens by the anti-*T. rhodesiense* (P) serum were common to those of the homologous antigen preparations.

The antigen with a high molecular weight found in " homogenates " corresponds to the nuclear antigen of Brown & Williamson (1964), since it is removed by centrifugation. Its apparent absence in the akinetoplastic strain of *T. evansi* is interesting, but difficult to explain as the above authors found that it was present in the nuclear fraction, but not in the kinetoplast-containing fraction of *T. rhodesiense*.

The question arises whether serum antibodies to common antigens are protective. It was first shown by Ehrlich (1909), and later confirmed by many others, that immunity is strain specific. Investigations by Seed (1963) and Watkins (1964) also suggest that protection induced by immune serum is variant specific. These are indications that antibodies to common antigens have no protective value.

The specific antigen identified in the *T. rhodesiense* (P) strain corresponds to the 4*S* antigen(s) of Brown & Williamson (1962, 1964) by virtue of its physical and biological properties. Their 1*S* antigens were not observed. In double diffusion tests the specific antigen formed a very broad, slightly curved band going through the antiserum well (Fig. 2). These are indications that it is highly concentrated and of slightly lower molecular weight than the antibody, which is consistent with the estimates of Brown & Williamson (1962). No evidence of more than one specific precipitinogen in a particular strain was obtained, unlike the four 4*S* antigens identified by Brown & Williamson (1962) and Williamson & Brown (1964). A possible explanation is that the precipitin lines are superimposed because of poorer separation in agar as compared to starch electrophoresis (Williamson & Brown, 1964). It is, however, significant that qualitatively the *T. rhodesiense* (P) strain retained its antigenic specificity over four years of regular passage as shown by double diffusion as well as agglutination tests.

Summary

Precipitinogens common to a number of *Trypanosoma* spp. were identified in double diffusion precipitin and immunoelectrophoretic studies. The *T. rhodesiense* strain used to produce the antiserum, which served as main indicator, was found to share antigens with a relapse strain derived from it, as well as with *T. equinum*, *T. equiperdum*, akinetoplastic and normal strains of *T. evansi*, and *T. lewisi*.

A specific antigen, which had remained qualitatively unchanged over four years of continual passage, was detected in the homologous *T. rhodesiense* strain by precipitin and agglutination tests.

It was impossible to distinguish between electrophoretic patterns of *T. rhodesiense* and *T. equinum* in starch gel, but *T. lewisi* was different.

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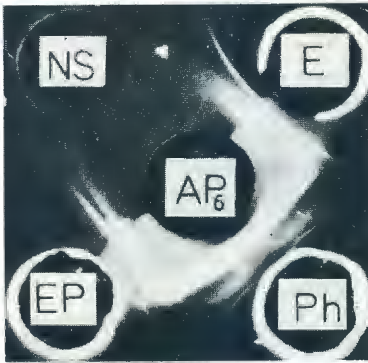

FIG. 1.—Precipitinogens in " homogenates " of *T. equinum* (E), *T. rhodesiense* parent (Ph), *T. equiperdum* (EP) and serum from uninfected rats (NS), identified by 15/6 rabbit antiserum (AP6)

FIG. 2.—Antigens in " cell sap " (Pcs) and " crude antigens " of *T. rhodesiense* parent (Pca), *T. equinum* (E), *T. rhodesiense* relapse (R), lyzed rat blood (B) and uninfected rat serum (NS). Antiserum as in Fig. 1 (AP6)

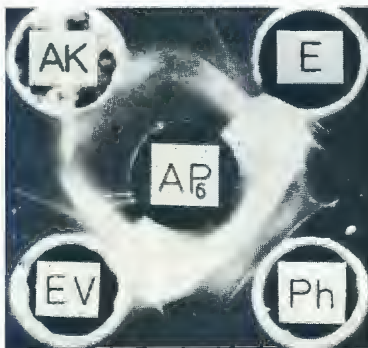

FIG. 3.—Antigens in " homogenates " of *T. equinum* (E), *T. rhodesiense* parent (Ph), *T. evansi* (EV) and an akinetoplastic strain of *T. evansi* (AK). Antiserum as in Fig. 1 (AP6)

FIG. 4.—Antigens in " homogenates " of (from left to right) *T. rhodesiense* parent strain (Ph) diluted to 50 per cent of its original concentration, undiluted *T. rhodesiense* (Ph) and *T. rhodesiense* relapse (R). Antiserum as in Fig. 1 (AP6)

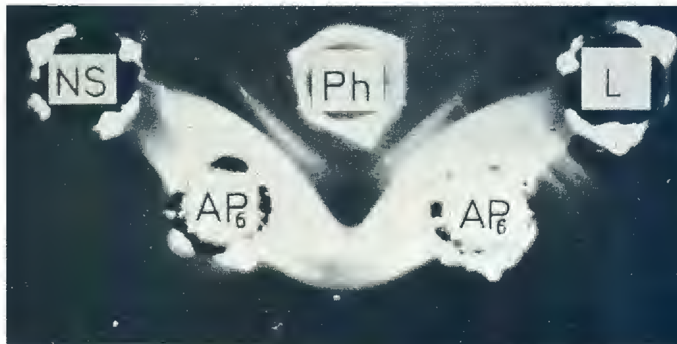

FIG. 5.—Antigens in serum from uninfected rats (NS), and " homogenates " of *T. rhodesiense* parent (Ph) and *T. lewisi* (L) as revealed by rabbit antiserum (AP6)

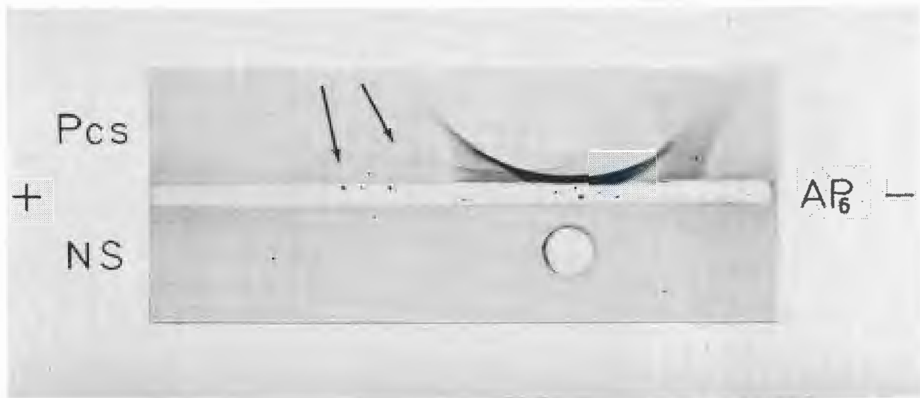

FIG. 6.—Immunoelectrophoresis of "cell sap" of *T. rhodesiense* parent (Pcs) and serum from uninfected rats (NS). Antiserum as in Fig. 1, etc. (AP6)

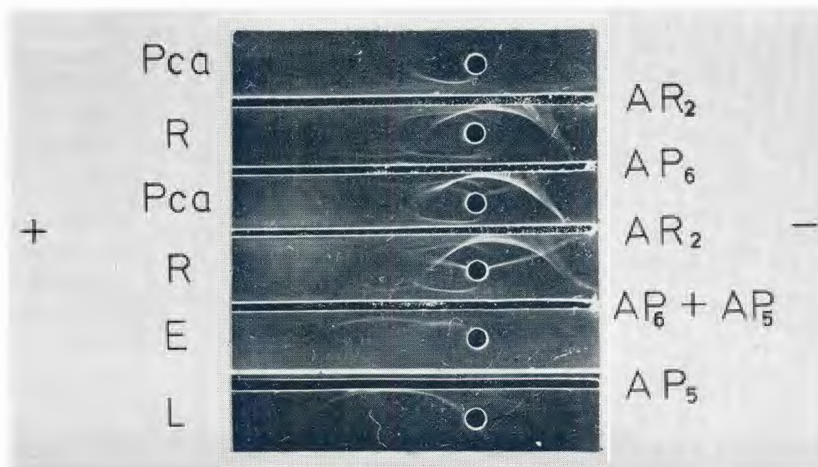

FIG. 7.—Immunoelectrophoretic study of "crude antigens" of *T. rhodesiense* parent (Pca) and *T. rhodesiense* relapse (R) strains, using homologous and heterologous antisera—AP6 = 15/6, and AP5 = 15/5 are homologous to Pca, and AR2 = 16/2 to R. *T. equinum* (E) and *T. lewisi* (L) are included for comparison.

Note that the line crossing the Pca well is due to a AR2/R precipitin reaction, with R from the adjacent run diffusing across; the line crossing the R well is likewise due to a AP6 + AP5/Pca reaction

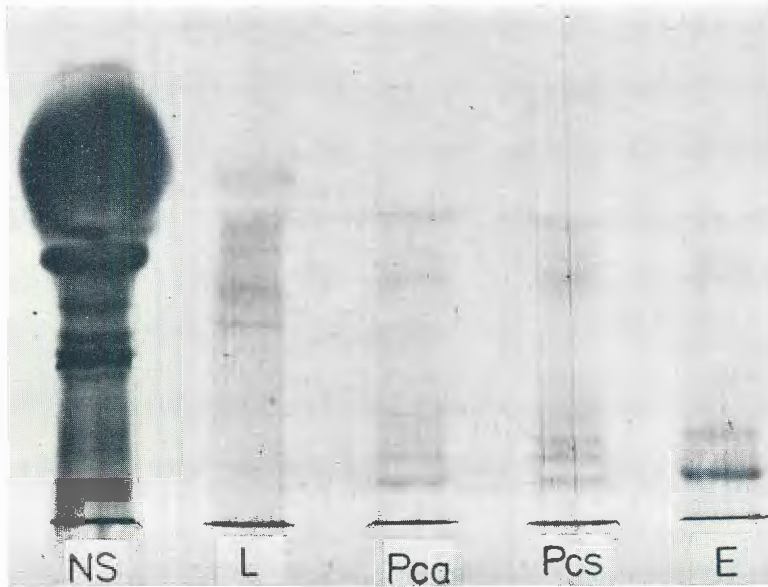

FIG. 8.—Zone electrophoresis in starch gel. From left to right, serum from uninfected rats (NS) is compared with "crude antigens" of *T. lewisi* (L) and *T. rhodesiense* parent (Pca), "cell sap" of *T. rhodesiense* parent (Pcs), "crude antigen" of *T. equinum* (E)