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Crossreacting determinants in variant-specific surface antigens of African trypanosomes

(glycoprotein purification/radioimmunoassay/immunofluorescence/*Trypanosoma brucei*/*Trypanosoma congolense*)

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ABSTRACT **A number of variant-specific surface antigens (VSSAs) were purified from clones of *Trypanosoma brucei* and *T. congolense* and tested for immunological crossreactivity. Anti-VSSA sera were clone-specific when tested by indirect immunofluorescence of living trypanosomes, but they were not clone-specific when tested by radioimmunoassay with purified ^{125}I-labeled VSSAs. In this double-antibody radioimmunoassay, every VSSA tested was precipitated by the homologous and all heterologous anti-VSSA sera. Any unlabeled VSSA could inhibit the heterologous precipitation reactions by 100%. The homologous precipitation reactions were effectively inhibited only by unlabeled homologous VSSA. Crossreactions between different VSSA molecules were also revealed by microcomplement fixation tests. The results confirm the presence, in VSSAs, of variable determinants specific to individual VSSAs and also show crossreacting determinants in all VSSAs tested, including those isolated from different species of trypanosomes. These results contrast with previous studies which failed to find evidence for immunological crossreactivity between different VSSA molecules. We suggest that the crossreacting determinants in VSSAs may represent common structural regions. The existence of such regions would have considerable implication for the development of theories and experiments concerning the mechanism of antigenic variation in African trypanosomes.**

The problems of developing a useful vaccine against African trypanosomes center on the ability of the organisms to undergo antigenic variation (reviewed in ref. 1). Antigenically distinct types have been observed by the use of the antibody agglutination reaction at intervals during the course of an infection (2). It is known that antibody against a single variant type of trypanosome can agglutinate that variant but not other variants arising from the original infecting population (3). The ability of trypanosomes to express variant antigens is associated with the presence of a surface structure, "the coat," a layer 12–15 nm thick overlaying the cytoplasmic membrane (4, 5). Evidence that the surface coat contains the variant antigens is reviewed in ref. 6.

Recently, by using the *N*-acylating reagent formylmethionine sulfone methylphosphate as a surface label, a glycoprotein that is the major constituent of the surface coat was successfully isolated from each of a number of clones of *Trypanosoma brucei* (7). The glycoproteins from different variant clones had the same apparent molecular weight (about 65,000 as determined by sodium dodecyl sulfate gel electrophoresis) but could be distinguished by their isoelectric points. An antiserum against a purified surface glycoprotein stained, by immunofluorescence, the surface of trypanosomes of the clone from which the glycoprotein was derived, but not other trypanosome clones. Immunization of mice with purified surface glycoprotein protected against challenge with a small number of homologous, but not heterologous, trypanosomes (7). No immunological crossreactions between the purified antigens were observed (7); neither were there any similarities in the extreme NH_2-terminal amino acid sequences of a small number of surface glycoproteins (8). A second surface labeling technique, ^{125}I-iodination catalyzed by lactoperoxidase, has been used in characterization of the surface coat of *T. congolense* (9). The variant-specific surface antigen (VSSA) of *T. congolense* was shown to have a slightly smaller apparent molecular weight than that of *T. brucei*.

In some respects it is surprising that immunological crossreactivity has not been observed between VSSAs. Although the data show that VSSA regions exposed on living trypanosomes are antigenically distinct, this is not necessarily the case with nonexposed parts of the molecule. Arguments for some conservation of structure between different VSSAs seem reasonable. For example, there might be a common region of the molecule responsible for membrane binding. The surface coat is the initial barrier between the organism and the external environment. Therefore, specific permeability properties may be required, which could depend on some conservation of the three-dimensional structure. It is our intention in this paper to reexamine whether antigenic similarities exist between isolated VSSAs of trypanosomes.

MATERIALS AND METHODS

Origin of Trypanosomes Used in Experiments. *T. brucei* 427 was isolated in 1960 from an early sheep infection, in Uganda (10). The history of this strain since then is described in ref. 11, and the origin of clones 049, 052, and 055 made from the 427 strain is described in ref. 7.

T. brucei EATRO 795 was isolated in 1964 from a cow, in Kenya. The designation 227 was assigned after a series of mouse passages. Clones A4 and 9C were derived from strain 227 by injecting a single trypanosome into the peritoneal cavity of lethally irradiated [900 rads (9J/kg)] mice. Clone 367 is a parasitemic population arising from a single metacyclic trypanosome in irradiated mice. The metacyclic trypanosome was obtained from *Glossina morsitans* infected with clone 9C bloodstream trypanosomes (courtesy of L. Jenni, Swiss Tropical Institute, Basel, Switzerland).

T. congolense "Transmara" strain was isolated from an infected cow near the Kenya–Tanzania border in 1966 (12). Clone X4 was derived from the strain in lethally irradiated mice (900 rads).

For the purposes of the present paper, trypanosomes are designated: *T. brucei* clones 049, 052, 055, A4, 9C, 367; *T. congolense* clone X4. Trypanosomes were routinely thawed from a stabilate [infected blood containing 7.5% (vol/vol)

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Abbreviation: VSSA, variant-specific surface antigen.

glycerol stored in liquid nitrogen] and inoculated into lethally irradiated mice (900 rads). The highly parasitemic blood was used to infect normal rats, from which trypanosomes were isolated 2–3 days later by described methods (13).

Lactoperoxidase-Catalyzed Surface ^{125}I-Iodination of Trypanosomes. Trypanosomes were iodinated by described methods (9), except that the H_2O_2 concentration in the reaction mixture was lowered to 5 μM.

Purification of Variant-Specific Surface Antigens. All surface glycoproteins were isolated as described (7), except for 049, which was provided by G. A. M. Cross (The Wellcome Research Laboratories, Kent, England). Isoelectric points of purified glycoprotein from *T. brucei* clones 049, 052, and 055 and *T. congolense* clone X4—7.5, 6.9, 8.2, and 6.6, respectively—have been reported (7, 9). The isoelectric points of VSSAs isolated from 9C, A4, and 367 trypanosomes were 5.5, 6.2, and 6.1, respectively.

Antisera. Anti-VSSA sera were made in rabbits by injection of the purified glycoproteins. Initial injections were divided equally among the footpads and contained approximately 10 μg of VSSA protein in 1 ml of Hanks' balanced salt solution mixed thoroughly with 1 ml of complete Freund's adjuvant. The second injection was given in two intramuscular sites 2 weeks later and consisted of the same amount of antigen mixed with 1 ml of incomplete Freund's adjuvant. The third injection had the same composition as the second and was given intramuscularly 2 weeks later. The rabbits were bled 7–10 days after the third injection, and the sera were collected and stored at −20°.

Antisera to rabbit IgG for use in the radioimmunoassay and for tetramethylrhodamine isothiocyanate conjugation were made in sheep. Two sheep were given 300 μg of isolated rabbit IgG (14) each, in balanced salt solution mixed with 2 ml of complete Freund's adjuvant, in multiple intramuscular sites. These injections were repeated twice at 2-week intervals, except that incomplete Freund's adjuvant was used. Sera were collected 1 week after the third injection and tested by immunoelectrophoresis and double immunodiffusion.

Radioimmunoassay. The procedure was similar to that described by others (15). Assays of antigen–antibody reactions contained normal rabbit serum (0.01 ml), ^{125}I-labeled VSSA (0.01 ml containing 1–4 ng of VSSA and 3×10^4 cpm), diluted rabbit antiserum to VSSA (0.01 ml), and 0.025 ml of TEN buffer (20 mM Tris·HCl/1 mM EDTA/10 mM NaCl/fraction V bovine serum albumin at 2 mg/ml, pH 7.6). This mixture was incubated for 3 hr at 37°, and then 0.1 ml of sheep anti-rabbit IgG serum was added and the tubes were incubated overnight at 4°. The tubes were centrifuged ($2000 \times g$, 30 min) at 4°, the resultant precipitate was washed twice with 0.5 ml of the same buffer, and its radioactivity was determined in a Packard Auto-Gamma scintillation spectrometer. The amount of sheep anti-rabbit IgG serum required (0.1 ml) was determined by using a trace amount of ^{125}I-labeled rabbit IgG with the various rabbit antibody dilutions. In those cases in which 0.01 ml of undiluted antibody to VSSA was tested, the normal rabbit serum was omitted from the assay.

Competition radioimmunoassays were performed by selecting an anti-VSSA serum dilution precipitating approximately 50% of the ^{125}I-labeled VSSA. Unlabeled VSSA glycoproteins were added just before the anti-VSSA serum and their ability to inhibit binding of ^{125}I-labeled VSSA was determined.

Dilutions of antibody and antigens were made in the same buffer containing fraction V serum albumin at 20 mg/ml. Chloramine-T-mediated iodination of VSSAs was performed as described (16) with 5 μg of protein and 1 mCi of ^{125}I for each VSSA.

Fluorescent Antibody Procedures. The IgG fraction of sheep anti-rabbit IgG serum was conjugated and the product was separated on DEAE (17). Those fractions having an absorbance ratio (280 nm/550 nm) between 2 and 3 were pooled.

Isolated trypanosomes (0.1 ml containing 5×10^6 live organisms were washed with Dulbecco's phosphate-buffered saline containing 10% fetal calf serum and then incubated for 30 min with dilutions of rabbit anti-VSSA serum (0.1 ml) in an ice bath. They were then washed three times, incubated for 30 min with fluorescence-labeled sheep anti-rabbit IgG (0.1 ml containing IgG at 0.3 mg/ml), washed three times, spread on a microscope slide, air dried, fixed with absolute ethanol, mounted with 50% glycerol, and examined. A Leitz Orthoplan microscope equipped with an Osram HBO-200 mercury vapor lamp and Ploemopak vertical illuminator was used. Trypanosomes were observed either under phase-contrast or with illumination and filter combinations set for rhodamine.

Complement Fixation. A quantitative microcomplement fixation procedure was selected for use, in order to conserve antigen (18).

Double Immunodiffusion. Antibody–antigen reactions were performed in 2% (wt/vol) Noble agar dissolved in 0.1 M Tris·HCl (pH 8.0) with a final concentration of thiomersalate of 1:10,000. Antisera were used undiluted and diluted 1:4 and 1:8; antigens were tested at approximately 0.5 mg/ml.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out by the method of Weber and Osborn (19), as modified by Cross (7). After electrophoresis, gels were stained for protein with Coomassie blue or sliced into 1-mm segments for measurement of ^{125}I-radioactivity.

RESULTS

Crossreactivity of VSSAs by Radioimmunoassay. Varying dilutions of rabbit antisera to purified VSSAs were incubated with ^{125}I-labeled VSSAs. Then, bound antigen was precipitated by sheep anti-rabbit IgG and radioactivity in the washed precipitate was measured. The radioactivity in the washed precipitate was compared to that in three control tubes containing: (*i*) labeled antigen precipitated by normal rabbit serum and sheep anti-rabbit IgG (the amount of labeled antigen precipitated by normal rabbit sera did not exceed 3%), (*ii*) labeled antigen alone, and (*iii*) labeled antigen precipitated by 10% (wt/vol) trichloroacetic acid (always greater than 90% of the total radioactivity). From these measurements, antiserum dilution was plotted against percentage of the total radioactivity in protein that was specifically precipitated. Fig. 1 *upper* shows such a plot for ^{125}I-labelled *T. brucei* VSSA 055 precipitated by using one homologous (anti-055) and three heterologous (anti-*T. brucei* 049, 052; anti-*T. congolense* X-4) sera. Although the homologous antiserum bound radioactive label most efficiently at the higher antiserum dilutions, all the heterologous antisera were also very effective. With these assay conditions, it was possible to precipitate more than 80% of labeled 055 antigen by using either anti-049 or anti-052 serum and 70% by using the anti-*T. congolense* X-4 serum. When less labeled antigen was used almost 100% of the labeled antigen was precipitated by all undiluted anti-VSSA sera. Fig. 1 *lower* is a similar plot, but in this case a single antiserum (anti-052) is shown precipitating one homologous (052) and two heterologous (049, 055) antigens. Some of the immunoprecipitates were solubilized in sodium dodecyl sulfate and aliquots were ana-

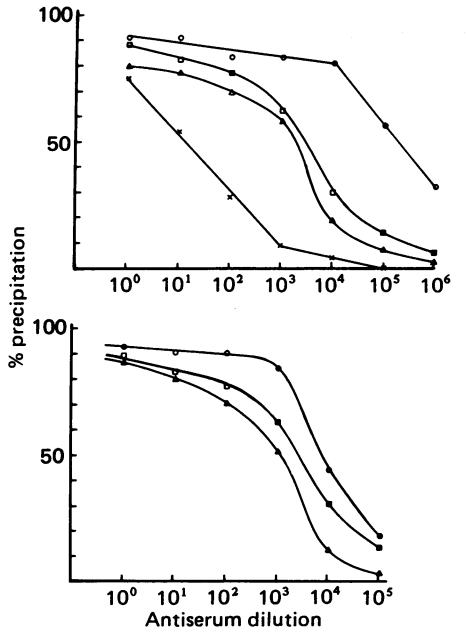

FIG. 1. Double-antibody radioimmunoassay plots for ^{125}I-labeled VSSAs and anti-VSSA sera. (*Upper*) ^{125}I-055 and anti-055 serum (○), anti-049 serum (△), anti-052 serum (□), and anti-*T. congolense* X4 serum (×). (*Lower*) Anti-052 serum and ^{125}I-052 VSSA (○), ^{125}I-049 VSSA (△), and ^{125}I-055 VSSA (□).

lyzed by gel electrophoresis (Fig. 2); the plots obtained demonstrate that radioactivity was present only in a peak of 65,000 apparent molecular weight.

These initial results were expanded to include every possible combination between antigens and antisera (six from *T. brucei*, one from *T. congolense*). The plots are too many to be presented here but, because they are all similar, two further examples are shown to illustrate the point. Fig. 3 shows a plot of ^{125}I-labeled 049 and 367 VSSAs precipitated by seven antisera, each raised against a single, different, purified VSSA. The homologous and all heterologous antisera bound the antigens. The amount of crossreaction between VSSAs and anti-VSSA sera was variable. This could be due to several reasons—e.g., variation in structure of crossreacting determinants, variation in immunodominance of crossreacting determinants compared to other parts of the VSSA molecule, or variation in potency of the anti-VSSA sera.

To show that the binding activity of immune serum was associated with immunoglobin, an IgG fraction was isolated from

Table 1. Relationship between crossreacting determinants of various VSSAs

	VSSA required for 50% inhibition, ng					
Antibody	367	9C	A4	055	052	049
Anti-367	6	(0%)*	(0%)*	(14%)*	(18%)*	(0%)*
Anti-052	5	12	9	3	15	5
Anti-X4	12	4	10	16	16	7

Each assay tube contained 4 ng of ^{125}I-labeled 367 VSSA. Antisera dilutions were selected to give 50% precipitation of labeled antigen.
* 50% inhibition was not achieved; the percentage inhibition obtained with 800 ng of VSSA is shown.

the anti-052 VSSA rabbit serum and from normal rabbit serum. Double-antibody radioimmunoassay showed that binding activity for homologous and heterologous antigens was present in the IgG fraction from anti-052 serum but not in the IgG fraction from normal serum.

Other experiments investigated inhibition of the precipitation reactions by unlabeled VSSAs. Three precipitation reactions were initially selected: one homologous combination (^{125}I-055 VSSA precipitated by anti-055 serum) and two heterologous combinations, one intraspecies (^{125}I-055 precipitated by anti-052) and one interspecies (^{125}I-055 precipitated by anti-*T. congolense* X4 serum). Antisera dilutions that precipitated approximately 50% of the labeled antigens were used and the reactions were inhibited by various unlabeled VSSAs. The homologous reaction was inhibited by as little as 6 ng of homologous antigen 055; but up to 600 ng of the heterologous antigens (049, 052) was ineffective. In contrast, both of the heterologous reactions were completely inhibited by 60–600 ng of any unlabeled antigen tested. These observations were extended by selecting other homologous and heterologous antigen–antibody reactions and testing for inhibition by unlabeled VSSAs (Table 1). Three to 16 ng of any unlabeled VSSA caused 50% inhibition of any heterologous reaction tested, but all homologous reactions were inhibited effectively only by the homologous antigen. Slight inhibition (less than 18%) of homologous systems by large amounts (800 ng) of heterologous antigen was occasionally observed.

These results show a crossreacting determinant or determinants in the trypanosome VSSAs tested, to which antibodies are produced by inoculation of the isolated molecules. Antibodies raised against one purified antigen will bind to any other purified antigen and binding may be inhibited by any third purified antigen, suggesting that the crossreacting determinant(s)

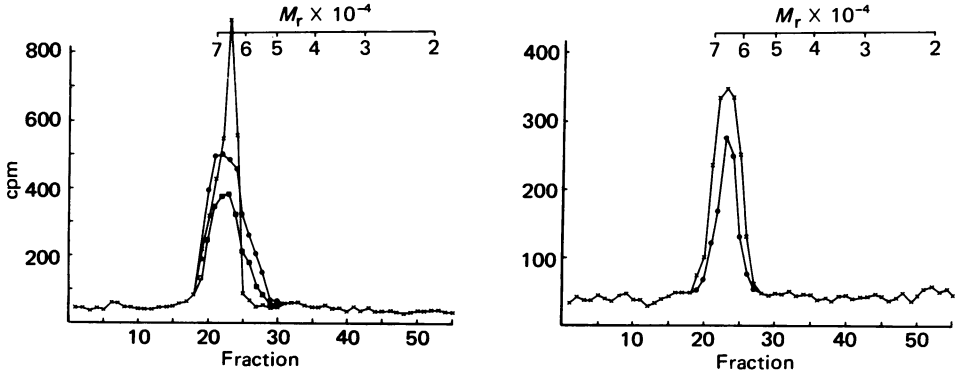

FIG. 2. Sodium dodecyl sulfate gel electrophoresis of VSSAs and solubilized VSSA–double-antibody immunoprecipitates. (*Left*) ^{125}I-055 (×); ^{125}I-055 precipitated with anti-055 serum diluted 1:1000 (○); and anti-049 serum diluted 1:1000 (□). (*Right*) ^{125}I-055 precipitated with anti-052 serum diluted 1:1000 (×) and anti-*T. congolense* X4 serum diluted 1:1000 (○).

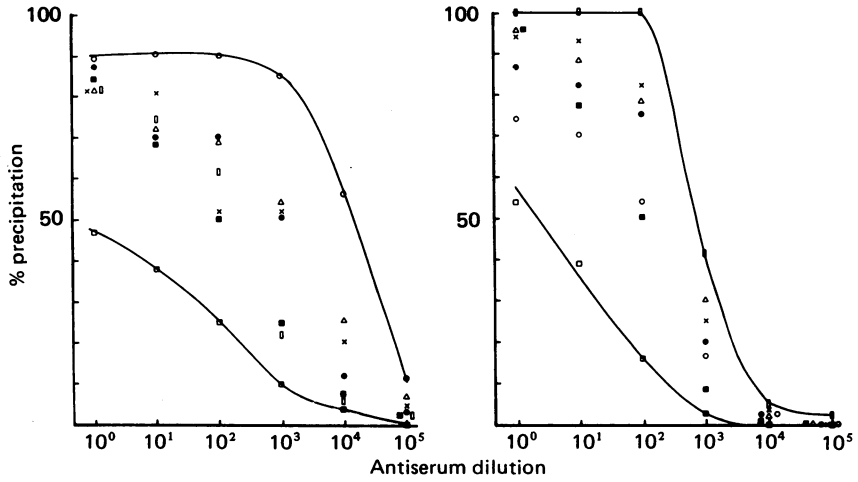

FIG. 3. Double-antibody radioimmunoassay plots for ^{125}I-049 VSSA (*Left*) or ^{125}I-367 VSSA (*Right*) and anti-VSSA sera: anti-367 (‖), anti-9C (■), anti-A4 (×), anti-049 (○), anti-052 (●), anti-055 (△), and anti-*T. congolense* X4 (□).

is similar in all cases. The homologous precipitation reactions are not effectively inhibited by any heterologous antigen. The likely reason for this is that antibodies raised against variable determinants predominate over those against the crossreacting determinants and, in the homologous system, most binding is due to these anti-variable determinant antibodies.

Radioimmunoassay with Supernatant Fluid from Trypanosomes Surface-Labeled by Lactoperoxidase-Catalyzed Radioiodination. An experiment was performed to demonstrate that the crossreactions observed with purified VSSA and antisera to purified VSSA could be shown by using a supernatant fluid from surface-labeled trypanosomes instead of purified VSSA. Clone 052 parasites (7×10^8) were surface-iodinated, washed, sonicated in 0.5 ml of H_2O (4°) and ultracentrifuged (240,000 × *g*, 60 min), and the supernatant fluid was dialyzed against 0.2 M NaCl/0.05 M Tris·HCl, pH 7.4 (4°). The radioimmunoassay was then carried out as described, except that the labeled supernatant fluid was substituted for the chloramine-T-labeled purified VSSA. Undiluted antiserum raised against the purified 052 glycoprotein precipitated 85% of the label (Fig. 4). Heterologous antisera raised against purified 055 and 049 glycoproteins precipitated about 30% of the label and anti-*T. congolense* X4 VSSA precipitated 9%. Precipitation of label occurred also at antisera dilutions of 1:10, but not at higher dilutions. The antisera appear to be less effective against the 052 VSSA in this assay than against the chloramine-T-labeled purified antigen because the specific activity obtained by the lactoperoxidase-catalyzed iodination technique was much lower. From knowledge of the yield of VSSA that would be expected from 7×10^8 trypanosomes, it was calculated that the activity of VSSA obtained in this experiment was 1–4 μg/30,000 cpm compared to 1–4 ng/30,000 cpm routinely used in the chloramine-T method.

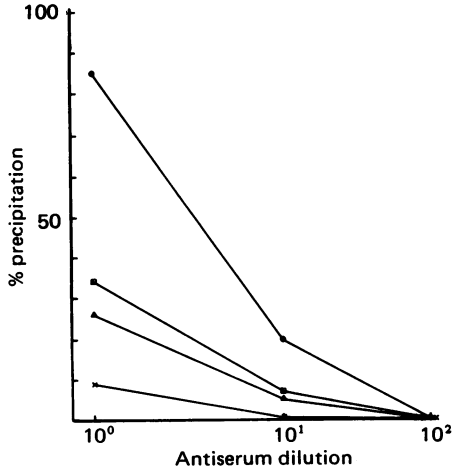

FIG. 4. Radioimmunoassay with a 240,000 × *g* supernatant fluid from 052 trypanosomes surface-labeled by lactoperoxidase-catalyzed radioiodination as antigen and antisera prepared against purified VSSAs, shown as ^{125}I label precipitated by: anti-052 serum (○), anti-055 serum (□), anti-049 serum (△), and anti-*T. congolense* X4 serum (×).

Immunofluorescence. Previous data shows that an antiserum raised against a purified surface glycoprotein will only stain trypanosomes of the clone from which that glycoprotein was derived (7). This finding was reinvestigated following the observation that crossreactions can occur when the purified VSSAs are used in radioimmunoassays. Each antibody prepared against a single purified VSSA was tested, by using indirect immunofluorescence, against every trypanosome clone. All antisera produced complete staining of the homologous trypanosomes at a dilution of up to 1:100. Further dilutions stained only particular regions of each trypanosome membrane. No heterologous staining was observed even with undiluted antisera. Anti-367 VSSA serum stained only 68% of the trypanosomes of this isolate, showing that 367 is a population of mixed antigenic type and that the glycoprotein isolated represents the predominant population. Other trypanosome clones were greater than 99% pure. Thus, by immunofluorescence with live trypanosomes, each antiserum was variant-specific, in contrast to the results obtained in the radioimmunoassay.

Double Immunodiffusion. All antisera were tested against six purified VSSAs in the Ouchterlony system. In each case a single line was obtained corresponding to the homologous reaction. No reactions were observed in the heterologous combinations at the concentrations tested except for a faint, slowly developing, line for anti-055 against 367 VSSA. Otherwise, as by immunofluorescence, each antiserum appears to be specific to a single VSSA.

Complement Fixation. This assay was performed to examine if crossreactions among different VSSAs could be observed by a second method. The results (Table 2) show that 367 and A4 VSSAs cause reduction in hemolytic complement on reaction with the homologous and a number of heterologous antisera, compared to control assays.

Table 2. Fixation of complement in homologous and heterologous reactions between VSSAs and anti-VSSA sera

Antibody	Antigen A4	Antigen 367	No antigen
A4	0.062	0.066	0.737
367	0.092	0.060	0.671
055	0.052	0.043	0.601
052	0.053	0.044	0.697
X4	0.251	0.210	0.560
NRS_1	0.631	0.601	0.721
NRS_2	0.607	0.591	0.647
NRS_3	0.627	0.628	0.676
NRS_4	0.629	0.653	0.671
No antibody	0.708	0.716	0.711

Shown as mean absorbance values at 413 nm on duplicate assays. Antisera were tested at a dilution of 1:50. Antigens were 1 μg per assay (final volume, 0.7 ml), 2 CH_{50} units of guinea pig complement was provided, and 5×10^7 sensitized sheep erythrocytes were used (18). NRS, normal rabbit serum.

DISCUSSION

The results described in this paper show that there are crossreacting determinants in six *T. brucei* and one *T. congolense* purified VSSAs (i.e., all that were tested). In view of the significance of this statement, one must consider carefully any possible artefacts that could produce the observed radioimmunoassay and complement-fixation results.

1. Purified VSSA from one trypanosome clone could be contaminated with VSSAs from other minor variants arising in the population. This argument may be countered as follows: (*i*) purified VSSAs such as 055 and 9C crossreact very well by radioimmunoassay and yet their isoelectric points are separated by more than 2.5 pH units, so cross contamination is unlikely because of the final electrofocusing step in purification; (*ii*) to explain the results described it would be necessary to postulate that every purified VSSA is contaminated with every other one tested, because there is crossreaction throughout the series (any combination of antigen and antibody). Each antiserum raised against a purified VSSA was specific to the clone of trypanosomes from which the glycoprotein was derived, by immunofluorescence, even with undiluted antisera. Crossreactions by immunofluorescence would be expected if a VSSA were contaminated with other VSSAs, giving rise to mixed antibody populations. Also, contamination of all clones of *T. congolense* with *T. brucei* is unlikely.

2. The purified VSSAs could be contaminated with a soluble protein that is not VSSA. Then the precipitation observed in the heterologous systems would be due to the contaminating impurity. Against this argument are the following: (*i*) all the VSSAs are isolated at different isoelectric points, and therefore it is unlikely that all are contaminated with the same protein impurity; (*ii*) all values for isoelectric points and molecular weights of purified VSSAs agree with previous data provided for trypanosome variant antigens (7) and with our data using lactoperoxidase-catalyzed surface radioiodination to identify VSSAs (gel electrophoresis of heterologous and homologous immunoprecipitates showed all radioactivity to be in a position corresponding to the variant antigen); (*iii*) it is possible to precipitate almost all the ^{125}I label in a purified VSSA by antiserum raised against another VSSA, and therefore the protein in the precipitate represents the major part of the labeled VSSA preparation and is not a minor, contaminating impurity.

It is interesting to note that crossreactions were not generally observed by using immunodiffusion in this system, and this perhaps is why these have been undetected previously. The reason for this result is not clear as yet. The lack of crossreaction by immunofluorescence is readily explainable if the crossreacting determinants are in the interior of the "coat" when the VSSA is in a normal conformation on the surface of live trypanosomes. Crossreactions in VSSAs were shown by radioimmunoassay and complement fixation. In other systems, methods based on similar principles have been used to investigate crossreactivity—e.g., in immunoglobulins (20) and viral proteins (15).

These results have significance regarding the future development of trypanosomiasis research. If crossreacting determinants in VSSAs represent similar structural regions, as seems likely, questions arise concerning the extent and intramolecular location of such regions. Studies to date showing extensive variation in amino acid composition between VSSAs (7) suggest that structural homology is not extensive. One possibility for the location of crossreacting determinants in VSSAs is a membrane binding sequence, perhaps including sugars. A second possibility has been suggested by our finding (unpublished) that many VSSAs are susceptible to endogenous proteolytic degradation to produce a series of fragments each differing in molecular weight by approximately 8000. It is tempting to speculate that there are a number of similar sites distributed at intervals throughout each VSSA molecule that are susceptible to proteolytic cleavage and represent the crossreacting determinants. To discriminate between these and other possibilities, a detailed structural analysis of VSSAs is required.

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