Cross-Reacting Protein in Sera of Rabbits and Hamsters Hyperimmunized with Phlebotomus Fever Viruses[1] (35288)

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In previous communications, we (1, 2) reported the results of immunodiffusion (ID) and complement fixation (CF) reactions obtained when hyperimmune mouse ascitic fluids (IAF) to 12 viruses of the Phlebotomus fever group (PHL) were reacted with the homologous and heterologous antigens. In the majority of cases, each reacted monospecifically with its homologous antigen preparation and indicated clearly the value of IAF for typing individual viruses of this group. It was thought desirable to try to produce antisera (AS) which would show broad reactivity (common antibodies) with some or all members of the group and thus be a valuable serological reagent for group identification. Toward that end, rabbits and hamsters were immunized to explore this possibility. When such hyperimmune sera were prepared and tested by ID, it was found to contain type-specific antibody as well as a cross-reacting antibody common not only to PHL group, but also to unrelated arboviruses. The purpose of the present study was to elucidate and characterize the cross-reacting substance.

Materials and Methods. Viruses. The 12 PHL viruses stated in the previous communication (2) were utilized. Other viruses used were as follows: (i) Eastern equine encephalitis (EEE) Mass. (VR55) MP 463; (ii) Western equine encephalitis (WEE) Calif. MP 28; (iii) dengue Type-2 (DEN-2) New Guinea "C" MP 26; (iv) West Nile (WN) strain B 956 MP 32; and (v) tissue culture passaged Coxsackie B-1 (Cox. B) Conn. 5 (VR28).

Antigens. Sucrose acetone (3) or alkaline extracts (4) of normal (NMB) and virus infected suckling mouse brains (ISMB) were used as antigens. Antigens were also prepared from suckling mouse brains of animals which had been inoculated with either 4% bovine albumin or 2% corn starch solution in phosphate buffered saline (PBS), pH 7.8.

Antisera. Six antisera were prepared in albino rabbits against each of the following PHL viruses: Sicilian (SIC); Naples (NAP); Chagres (CH); Icoaraci (ICO); Candiru (CDU); and Punta Toro (PT). Supernatant fluids obtained after centrifugation of 20% ISMB homogenates in PBS were used as immunizing antigens. The inoculum consisted of 2 ml of supernatant fluid emulsified in an equal volume of Freund's complete adjuvant (CFA). Each rabbit received 5 im injections at 10-day intervals. Serum samples were obtained before immunization, and prior to each injection, and 10 days after the last inoculation. Rabbits were also immunized in a similar manner with NMB in CFA.

Two antisera were prepared in golden Syrian hamsters against CH and PT viruses. The inoculum was similar to that used for rabbits except that the total volume of the virus–adjuvant emulsion was 2 ml. Each hamster received 5 ip injections and was exsanguinated 10 days after the last inoculation.

Immunodiffusion. The micro ID and absorption techniques previously described by us (2, 4) were used.

Fractional precipitation of serum proteins. Each antiserum was subjected to fractional precipitation with $(NH_4)_2SO_4$ by the method described by Pennell (5). The 34% precipitated serum fraction (F-1) and that of the 60% (F-2) were reconstituted in water and

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TABLE I. Agglutination or Lysis of Sheep Erythrocytes by Anti-Chagres Sera.

Test no.	Antisera[a]	Reagents added	Results	Titer
1	Rabbit	RBC[b]	Agglutination	1:64
2	Rabbit	RBC + C'[c]	Lysis	1:256
3	Rabbit (absorbed by RBC)	RBC + C'	No lysis	<1:2
4	Mouse	RBC + C'	No lysis	<1:2
5	Hamster	RBC + C'	No lysis	<1:2
6	NRS[d]	RBC + C'	No lysis	<1:2

[a] Heat inactivated at 56° for 30 min.
[b] Unsensitized sheep erythrocytes.
[c] Guinea pig complement.
[d] Normal rabbit serum (control).

dialyzed against physiological saline and tested by ID against the homologous and heterologous test antigens.

Tests for C-reactive protein. Goat anti-CRP (Hyland) was reacted with the untreated rabbit and hamster antisera. As a positive control, human serum containing CRP (Cappel) was used.

Heterophile antibodies. To determine whether the antisera contain heterophile antibody(s), serologic tests were carried out using sheep erythrocytes (RBC). Table I shows the antisera used after being inactivated at 56° for 30 min. They were mixed with RBC to detect agglutinating antibodies or in presence of guinea pig complement (C') to detect complement fixing (CF) antibodies. In addition the antisera after absorption by RBC were tested by ID against the homologous and heterologous viral antigens.

Results. Immunodiffusion reactions with treated and untreated sera. All rabbit and hamster sera, reacted essentially the same in every test. For this reason, examples of the reactions obtained with rabbit anti-Chagres serum after 5 injections are presented. All antisera (central well) were reacted with all test antigens (peripheral wells). However, since the ID reactions were essentially identical with all heterologous antigens, the schematic representations show reactions with the following antigens only: CH, SIC, WEE, NMB, WN, and NAP.

Figure 1A shows the reactions obtained when unabsorbed rabbit anti-Chagres serum was reacted with the test antigens. Two common precipitin lines with all test antigens were seen in addition to a type specific line with the homologous antigen only. Figure 1B shows the ID patterns observed when the same antiserum was absorbed by NMB antigen. The lines caused by NMB antigen were eliminated completely, whereas common lines with all other antigens remained. This finding indicated that the latter lines were not due to antibodies to mouse brain tissue. None of the preimmunization serum samples reacted in ID with any of the test antigens.

When the 34% $(NH_4)_2SO_4$ precipitated

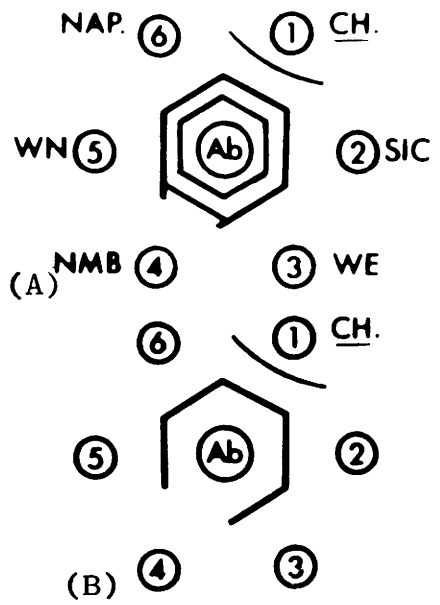

FIG. 1. Immunodiffusion patterns: Central wells contain rabbit anti-Chagres serum: unabsorbed (A) and NMB-absorbed (B). The peripheral wells 1–6 contain antigens of CH, SIC, WEE, NMB, WN, and NAP.

fraction (F-1), absorbed by NMB, was reacted with the test antigens the ID patterns observed were identical to those which appeared with the NMB-absorbed serum. No lines were observed with any of the antigens when the 60% $(NH_4)_2SO_4$ (F-2) was used. This indicated that the F-1 fraction contained all the cross-reacting protein and that it was most likely an immune globulin.

When goat anti-CRP was reacted with antisera prepared in rabbits against the various viruses no precipitin lines appeared except with the CRP positive control. This indicated that the viral antisera were devoid of detectable CRP or CRP-like substances. Hamster antisera also failed to react.

Tests for the role of heterophile-like antibody in causing the common precipitin lines. Table I summarizes the results of the series of tests carried out by mixing heat-inactivated antisera with sheep RBC in presence or absence of C′.

Tests 1 and 2 showed that the heat-inactivated rabbit anti-Chagres sera contained agglutinins and complement fixing antibodies to sheep RBC in titers of 1:64 and 1:256, respectively. Test 3 confirmed the result of Test 2, since after adequate absorption of the antiserum with RBC, no lysis occurred in presence of C′. Tests 4 and 5 indicated that neither mouse nor hamster anti-Chagres sera lysed RBC in presence of C′. Test 6 showed that normal rabbit serum (NRS) did not lyse RBC in presence of C′. These results indicated that only antisera prepared in rabbits contained heterophile antibody.

The common ID lines still appeared when the antiserum, absorbed by RBC and by NMB, was reacted with the test antigens. This clearly indicated that rabbit heterophile antibody(s), are not responsible for the common lines.

Immunodiffusion reactions of antisera absorbed by heterologous arbovirus antigens. Figure 2 shows that adequate absorption of the antiserum either by WN, DEN-2, WEE, EEE, or any heterologous PHL antigen, resulted in the elimination of the common lines leaving the specific line. These tests suggested that the common reacting protein might be antibody which appears in rabbit and hamster sera in response to a new or modified antigen present in ISMB.

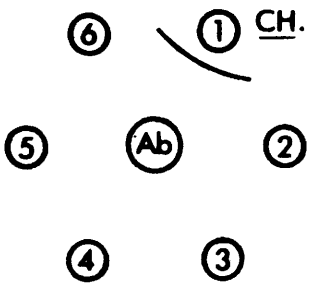

FIG. 2. Immunodiffusion patterns: The central wells contain rabbit anti-Chagres serum absorbed either by WN, DEN-2, WEE, EEE, or any heterologous PHL antigen.

Immunodiffusion reactions with nonarbovirus antigens. To determine whether the new or modified antigen in arbovirus ISMB may also result from nonarbovirus infection or from a pathological change in brain tissues as a result of trauma such as that caused by intracerebral inoculation of starch or the virus diluent, the following was carried out: Each antiserum was tested by ID against antigens of Cox. B ISMB, and brain antigens which were derived from animals inoculated with starch or virus diluent. No reaction appeared with any of these antigens.

Immunodiffusion reactions of rabbit anti-NMB with the test antigens. Unabsorbed rabbit anti-NMB gave precipitin reactions with all ISMB and NMB antigens. However, when the same antisera were absorbed by NMB, no lines appeared with any antigen. This showed clearly that the cross-reacting protein in rabbit sera was not due to immunization with mouse brain tissue and/or CFA.

Discussion. As mentioned in the introduction of this paper, hyperimmune sera prepared in rabbits and hamsters against some PHL viruses gave ID reactions not only with antigens of viruses of the same group, but also with those of antigenically unrelated arboviruses of Groups A and B. Antibodies to mouse brain tissue were readily eliminated by absorption of these antisera by NMB antigen preparation, and thus ruled out the possibility that the additional common ID lines were due due to antibodies to the mouse

brain tissue antigens. These reactions cannot be due to common arbovirus antibody(s) since investigators have not reported serological cross reactions among members of different arbovirus groups.

The following possibilities were considered and investigated; *i.e.*, the cross-reactions may have been due to one of the following: (i) a C-reactive protein-like substance similar to that found in human sera after pneumococcal or some bacterial infections (6, 7), or due to a CxRP-like substance found in rabbit sera following inoculation with certain bacteria, proteins or adjuvants (8); (ii) a heterophile or Forssman-like antibody as a result of immunization with ISMB. An analogous situation is the induction of Group A human blood RBC agglutinins in rabbits immunized with egg-grown vaccinia virus (9, 10). The presence of murine virus antibodies in the sera of rabbits and hamsters in response to these agents which may have been present in ISMB; (iii) a newly induced antibody(s) against a new or modified mouse brain "protein" due to viral infection. Analogous situations in virology are recognized, *e.g.*, induction of T-antigen(s) by oncogenic viruses, etc., or viral-induced enzymes which have been detected *in vitro* or *in vivo* systems.

It is unlikely that the cross-reactions were due to CRP-like substances. There was no detectable CRP-like antigen in any of the antisera when they were reacted with goat anti-CRP serum. Moreover, the antiserum fraction precipitated by 60% $(NH_4)_2SO_4$ gave no reactions with any of the test antigens. The 34% salt precipitated serum fraction however, contained the cross reacting "protein," indicating that the substance was most likely antibody. Further evidence, not presented, that it may be an immune globulin of the IGG type is that the untreated antisera and those treated with mercaptoethanol gave identical reactions. The possibility of its being a heterophile antibody was also investigated. The data presented indicated that despite the fact that rabbit antisera contained heterophile antibody, it was apparently not responsible for the cross-reactions because RBC-absorbed antisera gave identical ID reaction as the unabsorbed sera. In addition, hamster antisera showed identical ID patterns and contained the cross-reacting substance, yet did not contain heterophile antibody.

The possibility that the cross-reacting "globulin" may be an antibody to murine viruses present in suckling mouse brain, may also be ruled out by the following evidence: (i) most of the virus seeds prepared from ISMB and used in this study were found free of murine viruses (tests carried out by Microbiological Associates); (ii) if these virus seeds contained a murine virus, they would have stimulated the production of specific antibodies in mice. The data presented in the previous communications (1, 2) that mouse IAF and AS were monospecific to the homologous virus only, render this possibility very remote; (iii) rabbit anti-NMB sera did not contain this cross-reacting "protein"; (iv) absorption of antisera with NMB (if infected with murine virus) did not eliminate the cross-reacting antibody, but eliminated only antibodies to NMB.

One is left with the hypothesis that intracerebral inoculation of arbovirus into suckling mice induced a new or modified antigen in the brain tissues. When this was used to hyperimmunize rabbits and hamsters, it stimulated the production of antibodies to this new or modified "protein." Since absorption of each antiserum with any unrelated heterologous Group A or B arbovirus antigen eliminated the common lines, this hypothesis appears to be plausible.

The evidence that the new ID antibody formed appears to be specific for arbovirus and not due to an antibody in response to a nonspecific inflammatory reaction was shown in the studies where Cox. B and starch were used as inocula. These results seem to indicate the specificity of the reaction to arboviral infection. How broad this reaction would be if representatives of other groups of arboviruses or nonarboviruses were used, which are pathogenic in mice by the ic route (such as herpes, etc.) has not been determined.

The question remains whether this "new" antigen is one which is viral induced or simply the uncovering or modification of mouse brain giving rise to an heretofore unrecog-

nized new antigen. Had it been a *de novo* viral-associated common arboviral protein or enzyme one would expect the presence of antibody in mouse IAF or AS, if it was produced in sufficient quantity. Thus far, no such antibody has been found by CF, HAI, and ID testing procedures. This latter finding tends to support the hypothesis of a "uncovered" or modified mouse brain antigen which is not recognized by the mouse as foreign during the production of AS or IAF, but is recognized by the rabbit.

The implications of the study serve also as a word of caution to investigators who may use a similar system to produce arbovirus reagents in rabbits and hamsters. It also emphasizes the finding that heterophile antibody may be produced in rabbits following immunization with arbovirus ISMB. This latter antibody, however, is not responsible for the cross-reactions seen in these ID studies with the PHL viruses.

Summary. Hyperimmunization of rabbits or hamsters with infected suckling mouse brain of various Phlebotomus fever viruses led to the development of viral-specific and nonspecific cross-reacting antibodies which can be detected by immunodiffusion techniques. The cross-reacting antibody(s) was shown also to react with other nonrelated arbovirus antigens. Elucidation of the phenomenon showed that the reaction was not due to C-reactive protein, heterophile, or normal mouse brain antibody; rather it appears to be the result of a modified brain antigen present in the central nervous system following infection with arboviruses.

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