Elimination of Host Tissue Reactions in Immunodiffusion and Immunoelectrophoretic Studies of Arboviruses* (33655)

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Immunodiffusion (ID) and immunoelectrophoresis (IE) in agar gel were applied to the antigenic analysis of dengue viruses (1, 2). Rabbit antidengue sera proved to be superior, for this purpose, to those of mice, guinea pigs and hamsters (1). These antisera were prepared by a series of intramuscular injections of infected suckling brain homogenates (1). As expected, these antisera invariably contained antibodies to rodent tissue antigens as well as to viral antigens. When they were reacted against infected mouse brain (IMB) and against normal mouse brain (NMB) antigen preparations, multiple precipitin lines appeared with each. Since it was often difficult to discriminate with certainty between the reactions due to viral and nonviral antigens it was imperative to eliminate the precipitin lines due to reactions between the tissue antigens and their antibodies. The present communication describes the problems encountered to eliminate the undesirable reactions.

Materials and Methods. Viruses. The strains of dengue viruses and some other members of the same subgroup of group B arboviruses as well as their mouse brain passage levels were described previously (1).

Antigens. The following antigen preparations were used:

1. Crude supernates were prepared as 20% suspensions of infected and normal suckling mouse brain after centrifugation for 30 min at 13,200*g*. The diluent was Tris buffered saline (0.05 *M* Tris–0.15 *M* NaCl) at pH 8.0.

2. Similar crude supernates were prepared from infected and normal suckling hamster brains (IHB and NHB).

3. In an attempt to partially purify the above crude normal or infected supernates from the nonviral materials present in host tissues, the method described by Manson *et al.* (3) using flurocarbon–Freon 113, tricholorotrifluoroethane (DuPont de Nemours & Co.) was followed with some modification. Equal volume of the supernate and flurocarbon were mixed and homogenized in a chilled Lourdes blendor run intermittently for 1 min for 3 cycles. The homogenate then was centrifuged for 10 min at 7700*g*. The clear aqueous phase was saved and used as an antigen preparation and referred to as aqueous phase 1. Two further cycles of purification were carried out on a part of aqueous phase 1 to obtain aqueous phases 2 and 3.

4. Each crude supernate was partially purified by the method described by Warren *et al.* (4) using protamine sulfate (Sigma Chemical Company, St. Louis, Missouri).

5. Sucrose–acetone extracted antigens were prepared from normal and infected suckling mouse brains by the method of Clarke and Casals (5) except that after the final centrifugation, the supernatant fluids were used as antigens without lyophilization.

6. High speed pellets were prepared by

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centrifugation at 106,000*g* for 90 min from previously clarified 10% normal and infected suckling mouse brain suspensions. The pellet was resuspended in one twentieth of the original volume. The diluent was Tris buffered saline, pH 8.0.

Antisera. The immunization procedure for the rabbits was previously described (1), consisting of several intramuscular injections with adjuvant at 10-day intervals. The antisera employed in the tests here reported were prepared by injection of each of the types of antigens described above, except the high speed pellets.

Immunodiffusion and Immunoelectrophoresis. Both techniques were used as previously described (1, 2).

The test systems employed to determine whether the reactions between the antisera and the host tissue antigens were eliminated may be classified as follows:

1. Antisera prepared by injection of infected suckling mouse or hamster brain homogenates were reacted against the crude supernate antigen preparations from normal and infected suckling brain of the other species.

2. All the antisera prepared by injection of the crude or partially purified normal or infected suckling mouse or hamster brain homogenates were reacted against the various crude or partially purified supernate antigen preparations and the high speed pellets.

3. Specific absorption of antibodies to rodent tissue antigens. The absorbing antigen preparation was either NMB or NHB, depending on the animal source of brains used for immunization. Two methods were tried.

The first method was by mixing each antiserum undiluted and diluted 1:2 with an equal volume of 20% NMB or NHB in a centrifuge tube. Controls were set up by mixing each antiserum in a similar manner with an equal volume of the buffer diluent. The mixtures were held at 4° overnight or at room temperature for 2 hr and then centrifuged at 13,200*g* for 30 min. The supernates then were tested against normal and infected brain antigen preparations.

In the second method, the absorption was carried out in the agar gel of the ID or IE slides (absorption-in-gel) (6). Due to its importance, it is presented schematically in Figs. 1 and 2 for the ID and IE test, respectively. In Fig. 1A, the central well was filled first with the absorbing antigen preparation (20% NMB or NHB). The slide then was

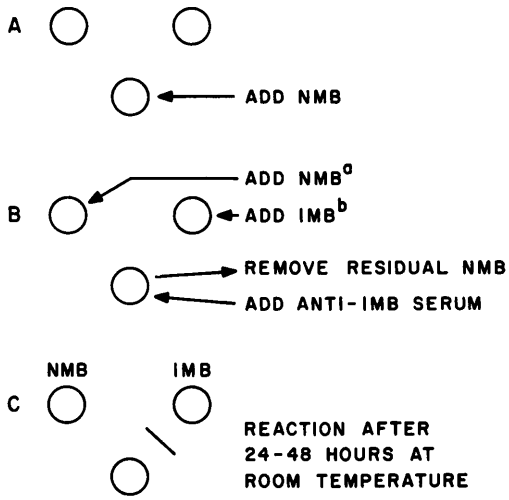

FIG. 1A and B. Schematic presentation of the steps followed in immunodiffusion for absorption of antiserum by NMB; (C) a hypothetical result. [a] Normal mouse brain antigen preparation; and [b] infected mouse brain antigen preparation.

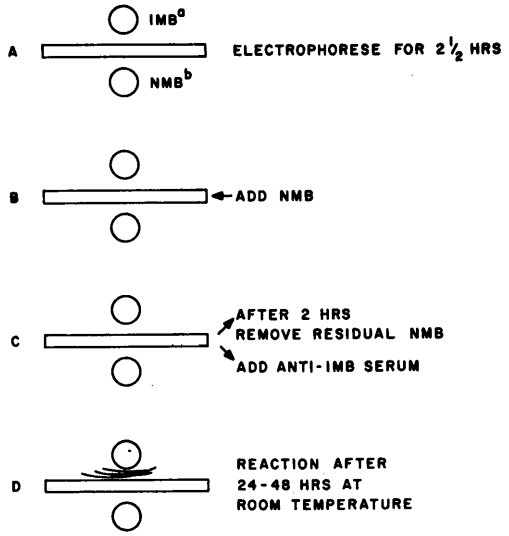

FIG. 2A, B, and C. Schematic presentation of the steps followed in immunoelectrophoresis for absorption of antiserum by NMB; (D) a hypothetical result. [a] Normal mouse brain antigen preparation; and [b] infected mouse brain antigen preparation.

TABLE I. Precipitin-in-Gel Reactions between Various Antisera and Various Normal Mouse Brain (NMB) and Normal Hamster Brain (NHB) Antigen Preparations.

Antisera against	Crude[a] NMB	Crude NHB	Partially[a] purified NMB	Partially[a] purified NHB	SA[b] NMB	NMB pellet
Crude infected mouse brain	+[c]	+	+	+	+	0[d]
Crude infected hamster brain	+	+	+	+	+	0
Partially purified infected mouse brain homogenates	+	+	+	+	+	0
Partially purified infected hamster brain homogenates	+	+	+	+	+	0
Partially purified NMB	+	+	+	+	NT	0
Partially purified NHB	+	+	+	+	NT	0

[a] Partial purification by either flurocarbon (aqueous phase 1, 2, or 3) or protamine sulfate.
[b] Sucrose–acetone extracted antigen.
[c] Positive precipitin reaction(s).
[d] No precipitin reaction.
[e] Not tested.

held for 2 hr at room temperature. The residual antigen then was removed and replaced by the antiserum to be absorbed (Fig. 1B). Each antiserum was used undiluted and diluted 1:2. Simultaneously, the peripheral wells were filled with the test antigen preparations including NMB or NHB in one of the wells to serve as control.

Figure 2 shows the steps used in IE. First the upper and lower wells of an agar slide were filled with normal and infected brain antigen preparations, respectively. The antigens electrophoresed for 2.5 hr (2) (Fig. 2A). The agar then was removed from the trough. It was then filled with 20% NMB or NHB supernate antigen (Fig. 2B). After 2 hr at room temperature, the residual absorbing antigen was removed from the trough and replaced by the test antiserum to be absorbed.

Fifty per cent NMB or NHB also was tried as the absorbing antigen in both ID and IE tests.

Results. Table I summarizes the results obtained by reacting the various antisera against the NMB and NHB antigen preparations. It is evident that antibodies to one rodent tissue antigen reacted with the rodent tissue antigens of the other species. It also shows that partial purification by flurocarbon (aqueous phase 1, 2, or 3) or by protamine sulfate failed to eliminate the rodent brain tissue antigens. It is of interest to mention that a line of nonspecific precipitation took place each time in ID agar slides between any protamine sulfate treated antigen preparations placed in two adjacent peripheral wells. Tissue specific antigen–antibody reactions also appeared with the sucrose–acetone extracted NMB antigen(s). However, antisera prepared against crude or partially purified IMB or IHB homogenates did not react except with the high speed pellet preparations obtained from IMB. The NMB pellet preparation produced no precipitin lines with any of the test antisera.

When 20% NMB or NHB crude supernate was used as absorbing antigen in either method of absorption (tube or absorption-in-gel), inadequate absorption of antibodies to rodent tissue antigen(s) resulted. Absorption in the centrifuge tube was carried out at both 4° overnight and at 24° for 2 hr. No difference was noted. However, when the cencentration of the absorbing antigen preparation was increased to 50%, adequate absorption by both methods occurred either with undiluted serum or serum diluted 1:2. In the second method (absorption-in-gel) 50% NMB was used to absorb antisera prepared against infected hamster brain and the absorption was completely successful. The reverse was carried out with the same result.

Figures 1C and 2D are schematic presenta-

tions of results obtained in ID and IE slides, respectively. Precipitin lines appear with infected brain antigen preparation, but not with normal brain supernate antigen.

Discussion. It was hoped that antibodies to mouse brain tissue antigens would not react significantly with hamster brain tissue antigen and vice versa. The results show clearly the extensive cross reactivity between the 2 rodent brain tissue antigens. Further evidence of a common antigen was obtained when antisera prepared against infected hamster brains were absorbed by NMB, also the reverse; adequate absorption occurred.

Failure to eliminate the tissue antigens by partial purification by flurocarbon or protamine sulfate, 2 methods extensively used for this purpose in virology indicate that the reacting antigens might be soluble in nature and are separable from the tissue cells following homogenization and centrifugation. The fact that the high speed centrifuged pellet (106,000*g*) of NMB did not react with the various antisera supports this assumption and indicates that the soluble antigens responsible for the reaction were in the supernate.

The two methods of absorption of antisera were adequate when 50% NMB or NHB was used as the absorbing antigen. The second method was originally developed by Dray and Young (6). It appears that this method has not been widely used. In the present studies this method was found simple, easy, time saving, sparing of reagents, and very effective. In antigenic analysis of California encephalitis viruses, in this laboratory, by ID and IE, this method also proved to be highly effective (7). In addition, its usefulness was proved in cross absorption tests using a heterologous virus antigen preparation to absorb an antiserum against another related virus and vice versa, to determine the extent of the antigenic relationship between two arboviruses (1, 2, 7). At the same time the heterologous IMB or IHB will absorb the antibodies to the rodent brain tissue antigen(s) just as did the NMB or NHB. This method of absorption should be useful in any other similar situation.

Summary. Host tissue reaction was a stumbling block in the progress of immunodiffusion and immunoelectrophoretic studies of arboviruses in this laboratory. Partial purification by flurocarbon or by protamine sulfate, of the infected rodent brain tissue homogenates used as test antigens or as inocula into rabbits to prepare antisera failed to remove the tissue antigen reactions. Antisera prepared against infected mouse brain or normal mouse brain reacted with normal hamster brain and vice versa. However, two methods were successful in eliminating the reaction: (i) Pellets obtained from clarified infected mouse brain homogenates following ultracentrifugation at 106,000*g* was free from rodent brain antigen but contained reacting viral antigens. (ii) Absorption of antisera either by 50% normal mouse brain or normal hamster brain eliminated the antibodies to the rodent brain tissue antigens. Two methods accomplished this latter—conventional tube absorption or absorption-in-gel. The latter is described in detail and found to be highly effective, simple, easy, time saving, and sparing of reagents. It is now used routinely in this laboratory.

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