Use of an Antiglobulin Plaque Neutralization Test among Group A Arboviruses* (33641)

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Although antiglobulin tests have been used with several different kinds of microorganisms (1, 2), they have only recently been applied to the detection of viral antibody (3–6). Ashe and Notkins (3) and Notkins *et al.* (4) demonstrated with herpes simplex virus and lactic dehydrogenase virus, that the sensitivity of detecting the reactivity of antibody with virus by conventional tests can be enhanced if the incubation of the virus with viral antibody is followed by addition of antiglobulin that was made against the viral antibody.

In 1967, our laboratory reported briefly on the detection of antibody by an antiglobulin plaque neutralization (AGN) test in a special case of a protection gradient induced in mice after a single immunization with a living, attenuated variant (E_m) of eastern equine encephalitis (E) virus (7). No conventional complement-fixing (CF), hemagglutination-inhibiting (HI), or neutralizing (N) antibody, with or without added accessory factors was detectable in the mice. The purpose of the present paper is to present data on a comparison of the AGN test with a standard plaque neutralization (N) test to detect homologous antibody against several group A arboviruses in infected mice.

Materials and Methods. Virus strains. The following viruses were used: (i) the Trinidad strain of Venezuelan equine encephalitis (V) virus (8); (ii) an attenuated, small-plaque variant of V called T (8); (iii) the Louisiana strain of eastern equine encephalitis (E) virus (8); (iv) a mutant of E, called E_m, which was obtained by Dr. A. Richter in our laboratory after nitrous acid treatment of E. Compared with parent E, the mutant forms minute plaques on chick embryo (CE) cells and is of lower virulence for 8- to 12-g mice after injection by the subcutaneous (s.c.) or intraperitoneal (i.p.) routes; and (v) the AR 339 strain of Sindbis virus (9). All virus seeds were made in CE cells and contained concentrations between 10^8 and $10^{9.5}$ pfu/ml.

The fluids used for comparing the AGN and N tests consisted of the following: (i) serum or ascites fluids collected 14–21 days after s.c. injection of 10- to 14-g mice with 10^4 pfu of E_m (nonlethal infection); (ii) serum or ascites fluids obtained at various intervals after intracerebral inoculation of 10- to 14-g mice with 10^5 suckling mouse LD_{50} of Sindbis virus (nonlethal infection); (iii) serum or ascites fluids collected at intervals following injection of 10- to 14-g mice by the i.p. route with 100 pfu of T (nonlethal infection); (iv) pooled serum collected from 5 mice daily after i.p. injection with 10^4 $IPLD_{50}$ of E (lethal infection). The method for using Sarcoma 180 cells to obtain large (e.g., 10–15 ml) volumes of antibody-containing ascites fluid from mice has been described by Tikasingh *et al.* (10).

For certain purposes, antibody fluids were absorbed with viruses to remove greater than 90% of the antibody. Usually a 1:10 dilution of the antibody-containing fluids (N titer> 1:1000) was made in beef heart infusion broth containing 0.1% bovine serum albumin fraction V (BHI-BSA) and incubated at 37° for 30 min with equal volumes of 10^7 pfu of either T or E_m. The fluids were then centrifuged at 105,000*g* for 1.5 hr to remove the virus–antibody complexes. The supernatant fluid was absorbed again in a similar fashion, and the second supernatant was frozen and stored at −70° until used.

* In conducting the research reported herein, the investigators adhered to "Guide for Laboratory Animal Facilities and Care" established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources NAS-NRC.

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TABLE I. Standard Plaque Neutralization Test and Antiglobulin Plaque Neutralization Test.

Standard	Antiglobulin
Constant virus + antibody dilutions	Constant virus + antibody dilutions
↓ incubation	↓ incubation
Assay for remaining virus	Add antiglobulin
	↓ incubation
50% end point	Assay for remaining virus
	50% end point

Cell cultures and virus assay. The usual methods for cultivation of CE cells and for assaying the viruses by a monolayer plaque technique have been described (8).

The AGN and N tests. Table I briefly outlines the routine procedures used for the standard plaque neutralization (N) test and the antiglobulin plaque neutralization (AGN) test. Between 10^5 and 10^6 pfu of virus contained in 0.2 ml of BHI (with or without BSA)[1] or in 1/40 normal ascitic fluids supernatant (NAF) were incubated at 37° for 45 min (or room temperature, 1 hr) with 0.2 ml of doubling dilutions of antibody. For the standard N test; 2- or 10-fold dilutions of this mixture were made, depending on the precision required, and assayed. A modification of the N test that was frequently included for more direct comparison with the AGN test included a second incubation with control goat antihuman gamma globulin at the same dilution as the goat antimouse gamma globulin used in the AGN test. The results by either N test, however, were not significantly different when the dilutions were taken into account. For the AGN test, a second incubation was included after the initial virus–antibody incubation with 0.2 ml of a 1:5 or a 1:10 dilution of goat antimouse gamma globulin[2] in BHI or 1/40 NAF. In developing the test, a number of controls were used. These included: (i) preinfection "normal" mouse serum or normal ascites fluids in the place of the antibody-containing fluid; (ii) antihuman gamma globulin in the place of antimouse gamma globulin; and (iii) a different group A virus in the place of the homologous virus and which is known not to be neutralizable by the homologous antibody. A fourth control was used only rarely. It consisted of using the supernatant obtained after low speed centrifugation of the precipitate resulting from the incubation of a 1:2 dilution of mouse serum or ascites fluid with a 1:3 dilution of goat antimouse gamma globulin. The antibody activity in such supernates was reduced more than 95% compared with untreated controls.

None of the first 3 controls used lowered the virus titer significantly, except in a rare case of a nonspecific inhibition present in mouse fluids up to 1:5 dilution. The end points of both N and AGN tests are the dilutions of antibody that produce a 50% reduction in plaque numbers compared with controls. A 2- to 4-fold increase in sensitivity of the AGN or N tests could usually be achieved if after preliminary experiments were done, virus dilutions were chosen so that 30–100 surviving pfu would be assayed to determine the 50% reduction in plaque numbers by an antiserum (5).

[1] The BSA is used with viruses that are labile at 37°.

[2] Mann chemicals, New York. The goat antimouse gamma globulin was ordered as precipitin type antibody free of preservative which usually has a titer between 1:16 and 1:32 by the Ouchterlony method at Mann. Goat antimouse serum in limited tests has been found to be as satisfactory as antimouse gamma globulin.

TABLE II. Comparison of AGN and N Tests on Sera and/or Ascites Fluids from Infected Mice.[a]

		Titer[c] on indicated day following immunization								
Immunizing virus	Test	0	2	4	6	8	10	12	14	16
E_m[b]	AGN[b]	<10	—	—	—	20	80	—	160	320
	N[b]	<10	—	—	—	<10	<10	—	<10	<10
T[b]	AGN[b]	—	—	—	—	40	—	160	—	640
	N[b]	—	—	—	—	<10	—	10	—	40
Sindbis	AGN	<10	<10	10	160	—	640	—	—	—
	N	<10	<10	<10	<10	—	20	—	—	—
E	AGN	<10	<10	20			Lethal infection			
	N	<10	<10	<10						

[a] Serum or ascites fluids rarely differed in titer by more than 1 doubling dilution when sample was collected on the same day.

[b] The respective parent viruses were used in the *in vitro* N and AGN tests.

[c] Reciprocal of dilution that reduced plaques to 50% of controls. Estimates are to highest doubling dilution tube when extrapolation between doubling dilutions are necessary.

Results. The results of comparing the two tests are shown in Table II. It is clear from these results that the AGN test is superior to the N test in all cases; it detects antibody sooner and/or to higher titer. In the case of infection with E_m, no antibody was detected by the N test, while antibody was detected in substantial titer by the AGN test. This has been the case in all 5 of 5 separate infections of mice with E_m, where peak titers detectable by the AGN test varied from 1:80 to 1:1280 in the absence of antibody detectable by the N test. The AGN method was also superior to the N method when testing various hyperimmune ascites fluids, but the differences were generally of a lower magnitude. They ranged from zero to 10-fold but most were between 2- and 4-fold. The hyperimmune antisera tested were to Venezuelan equine encephalitis virus, Sindbis virus, western equine encephalitis virus, and eastern equine encephalitis virus. The immunizations involved the use of β-propiolactone-killed virus, living virus or combinations of both; all antisera contained antibody concentrations higher than 1:1000 as measured by the standard N test.

The objectives of the next experiment were 2-fold: (i) to absorb the hyperimmune fluids with virus in order to reveal or enrich the antibody population of presumably lower avidity (11), and (ii) to test whether the neutralizing activity of this antibody population was enhanced by antiglobulin. Those antibody-containing fluids against V and E viruses which showed little difference in titer between the N and AGN tests were absorbed twice with Millipore-filtered preparations of small-plaque variants of the respective parent strains. The variants were used so that any small amounts of virus remaining in the fluids after absorption could be easily distinguished from the parent viruses that were used subsequently in the comparative tests carried out with the absorbed fluids. The results of this experiment (Table III) show that for 3 of the 4 antisera tested, antiglobulin enhanced the neutralizing antibody population that remained after virus adsorption more than it enhanced the antibody in the unabsorbed population. The increase in titer of at least 2 of the 3 antisera (V no. 1 and E no. 1) are considered significant because the results were reproducible in replicate tests. Such results support but do not prove the notion that antibody populations which are less avid are more susceptible to enhancement by antiglobulin than those that are more avid (12, 13).

A variation of the idea in the preceding experiment was tried by adding antimouse gamma globulin to the infectious virus remaining in the supernatant that was obtained after centrifugation of the virus anti-E

TABLE III. Comparison of AGN and N Tests on Virus-Absorbed and Nonabsorbed Hyperimmune Ascites Fluids.

Anti-serum	Nonabsorbed titer[b]		Virus-absorbed[a] titer[c]	
	AGN	N	AGN	N
V no. 1	1:2000	1:1000	1:16	<1:4
no. 2	1:8000	1:8000	1:128	1:64
E no. 1	1:8000	1:8000	1:128	1:32
no. 2	1:16,000	1:8000	1:512	1:512

[a] Each hyperimmune ascites fluid was absorbed twice with the homologous small-plaque variants (T, E_m) of V and E viruses, respectively. The N and AGN tests were carried out subsequently with the parent strain of each variant.

[b] Doubling dilutions were made beginning at 1:500.

[c] Doubling dilutions were made beginning at 1:4.

serum absorption mixture. The virus titer decreased by 1 $\log_{10}$ compared with controls (e.g., antihuman gamma globulin, BHI-BSA), showing once again that apparently infectious virus–antibody complexes were present in which neutralization could be demonstrated only by the addition of antiglobulin (3, 15).

Discussion. The practical use of antiglobulin added to various kinds of antigen–antibody complexes to enhance the sensitivity for detecting antibody is well documented as indicated above. Only recently has it been used for the detection of intact viral neutralizing antibodies or even of the active antibody fragments (3). In addition, Notkins *et al.* determined which fragments of the antiglobulin are active (14). In the cases reported by others, and from the results presented in this paper with group A arboviruses, it is clear that in a variety of infections, the AGN technique permits the detection of neutralizing antibody where none could be detected by standard N techniques, or the AGN technique detected antibody sooner and to higher titers than the N technique (Table II). Furthermore, it appears that the neutralizing activity of certain antibody populations can be enhanced by antiglobulin more than others (Table III), using the same virus.

The reasons why the addition of antiglobulin to virus–antibody combinations should enhance neutralization are not entirely clear. It has been postulated that antiglobulin somehow stabilizes the attachment of less avid antibody to the virus (13), or forms more or better bridges between the antiviral antibody molecules on the virus particles and thus blocks critical infective sites on the virus (3). These authors and others showed by neutralization kinetics that sensitized virus was more resistant to subsequent neutralization by antiviral antibody than was unsensitized virus (13, 15, 16). Antiglobulin against the antibody on the sensitized virus is able to neutralize the previously un-neutralized but "sensitized" virus–antibody complexes (3). Thus, the so-called "persistent fraction" which remained after neutralization had leveled off in a typical neutralization reaction (17), could be neutralized by adding antiglobulin (3, 15).

Recently, Wallis and Melnick (18) reported that the "persistent fraction" was due to virus in aggregates which could not be reached by antibody. They showed that preparations of several different viruses filtered so as to remove aggregates showed no "persistent fraction" whereas their unfiltered counterparts did, using the same antibody preparation at a given concentration. They also showed that various antisera had higher titers when tested with filtered virus preparations than with unfiltered preparations. In preliminary experiments in our hands, filtered preparations of our arboviruses carried out by the procedures recommended by Wallis and Melnick (18) were no more effective than unfiltered preparations in detecting antibody in E_m-infected mice by a typical N technique in 5 different experiments where the AGN technique on unfiltered preparations detected antibody titers ranging from 1:80 to 1:1280. However, the AGN titers were increased 4-fold, in the 2 cases that were tested, when filtered virus preparations were used. These results were reproducible in 3 tests on the same virus preparations, but the tests were only carried out with anti-

bodies made against E_m (Brown and Elsner, unpublished).

It is possible that filtered virus preparations and the use of antiglobulin would probably provide, for practical purposes, the most sensitive test for neutralizing antibody.

Summary. Among several infections in mice, both lethal and nonlethal, it was found that an antiglobulin plaque neutralization test was superior to a standard plaque neutralization test for the detection of antibody. In most cases, the antiglobulin plaque neutralization test detected antibody sooner and to higher titer than the standard plaque neutralization test. In one of the nonlethal infections, the antiglobulin plaque neutralization test detected antibody concentrations ranging from 1:80 and 1:1280 where it was otherwise undetectable by a standard plaque neutralization test, complement-fixation test or hemagglutination-inhibition test. In certain hyperimmune sera there was sometimes little difference in the results of the two tests, but absorption of the antisera with viruses revealed significantly higher titers by the antiglobulin plaque neutralization test than the standard plaque neutralization test in the remaining antibody population in at least 2 of 4 sera examined. In addition, infectious virus–antibody complexes which remained after absorption of antibody could be neutralized by the addition of antimouse gamma globulin but not by control fluids.

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