Labile Serum Factor and Its Effect on Arbovirus Neutralization

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Received for publication 24 September 1970

Enhancement of neutralization of Sindbis, Venezuelan equine encephalitis, Eastern equine encephalitis, Western equine encephalitis, and St. Louis encephalitis viruses by labile serum factor (LSF) in human serum and plasma was demonstrated. Human serum and plasma could be diluted 1:8 and 1:16 and still retain some LSF activity. Satisfactory storage temperatures for retention of LSF activity were −20 or −56 C. Repeated freeze-thaw cycles of serum did not alter LSF activity, but the activity was completely eliminated by heating at 56 C for 5 min. LSF of human serum equally enhanced neutralization by Sindbis immune mouse and rabbit sera; these results suggest a lack of species specificity. Rehydrated lyophilized guinea pig complement did not restore LSF activity to heated human plasma. Serum components responsible for LSF activity were not dialyzable. Discovery of fresh serum without LSF activity established the need to pretest all sera used as LSF sources.

The observations of many investigators have established without doubt that fresh serum enhances neutralization of a variety of viruses by homologous antisera (1, 4, 5, 7, 8, 12, 14-22; R. Pollikoff and M. M. Sigel, Bacteriol. Proc., p. 105-106, 1952). Since the component of fresh serum that enhances neutralization is heat-sensitive, it is commonly known as labile serum factor (LSF).

Despite the interest of many workers in LSF over the past 25 years, many practical questions applicable to its effective laboratory use still remain unanswered. The present studies were carried out to provide information of such practical nature as the effects of dilution, storage temperature, freeze-thaw cycles, and animal species homology on LSF activity. The basic system used to detect LSF activity was the neutralization of Sindbis virus by immune rabbit serum. Virus neutralization was measured by plaque assay in primary duck embryo cell cultures.

**MATERIALS AND METHODS**

**Virus.** The strain of Sindbis virus used (AR-1055) was passed 10 times in newborn mice by the intracranial (ic) route. The Fleming strain of Western equine encephalitis (WEE) virus was passed similarly more than 100 times, and Eastern equine encephalitis (EEE) virus (NJO strain) was passed six times. These viruses were prepared as 20% suspensions of suckling mouse brain in phosphate-buffered saline containing 4.0% bovine plasma albumin, pH 7.8. After lyophilization, these virus seeds were stored at −20 C.

St. Louis encephalitis (SLE) virus (strain TBH-28) was passed three times in suckling mice by the ic route and one time in HeLa cells. The SLE virus seed consisted of HeLa cell culture fluid harvested at the peak of cytopathic effect (CPE). An attenuated vaccine strain (TC-83) of Venezuelan equine encephalitis (VEE) virus was used. Seed virus of VEE was prepared in primary guinea pig heart cell cultures; in total, 84 passages were made in these cells. Fluid was harvested from these cultures at the peak of CPE and used as virus seed. Wet-frozen seeds of SLE and VEE viruses were stored at −56 C.

**Serum and plasma.** Immune rabbit sera were produced in New Zealand white rabbits by multiple injections of mouse brain vaccine. Infectious brain tissue was used for Sindbis, EEE, WEE, and SLE vaccine; however, the VEE vaccine was inactivated with beta-propiolactone. Sera and plasma from human donors were screened for neutralizing antibody to several viruses including Sindbis, VEE, EEE, WEE, and SLE. Serum and plasma samples free from antibody to these viruses were dispensed in vials and stored at −56 C as soon as possible after collection. Sindbis-immune mouse ascitic fluid was prepared by the method of Tikasingh et al. (20). The diluent used for serum, plasma, and ascitic fluid samples was 1 × Hanks balanced salt solution (HBSS) as described by Hanks and Wallace (10).

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Freezing and thawing serum. To determine the effect of freezing and thawing, serum in a plastic centrifuge tube was frozen by immersion in a mixture of dry ice and ethanol for 10 min and was then thawed rapidly in a water bath at 37°C. Ten such freeze-thaw cycles were carried out on the same container of serum, and a sample to be used for LSF tests was removed at each thawing. The pH of each test sample was determined.

Dialysis of serum. Human serum with known LSF activity was dialyzed at 4°C for 18 hr against 200 volumes of physiological saline. The dialyzed serum and a sample held at 4°C for 18 hr without dialysis were then assayed for LSF activity by using the Sindbis virus neutralization system.

Growth medium for cell cultures. Growth medium for duck embryo cells was supplied by Gerald Taylor, Tissue Culture Unit, Center for Disease Control. It consisted of a balanced salt solution base with 0.5% lactalbumin hydrolysate, 0.1% glucose, 5.0% newborn calf serum (heated at 56°C for 30 min), penicillin, streptomycin, and mycostatin.

Cell cultures. Primary cell cultures of embryonic duck cells were prepared from 13-day-old Pekin duck embryos by using a procedure similar to one described by Hsiung (13). Stock 10% cell suspensions were stored at 5°C until used. This suspension could be stored for 5 days without medium change and for 10 days with a change of medium every 2 days.

For plaque assay, the stock cell suspension was diluted 1:20 with growth medium. One-ounce (ca. 30 ml) prescription bottles (Sani-Glas), with rubberlined caps, were planted with 4 ml of cell suspension. Cultures were grown for 2 days at 35 to 37°C before inoculation.

Plaque assay procedure. Spent medium was discarded from confluent cultures, and each bottle was inoculated with 0.1 ml of virus suspension. Two cultures were inoculated with each virus dilution and were incubated at 35°C for 1 hr for virus adsorption.

To each bottle was added 4 ml of agar overlay medium. This medium contained the same ingredients as those described by Hsiung for chick embryo cell cultures (13). Bottles were incubated at 35°C. Plaque-forming unit (PFU) titers were based on the mean plaque number in all bottles containing 10 to 100 plaques.

Neutralization test. Tenfold dilutions of virus were made both in unheated serum and in serum that had been heated to 56°C for 30 min. Normal control and immune test sera and ascitic fluids were diluted 1:10 with HBSS and dispensed into tubes. These were heated at 56°C for 30 min to reduce native LSF to uniform levels. An equal volume of diluted virus was added to each serum or ascitic fluid sample, and these mixtures were incubated at 37°C for 1 hr. Two cell cultures were inoculated with 0.1 ml of each serum-virus mixture.

RESULTS

Effect of dilution on LSF activity of human serum. Sera from two human donors were diluted in HBSS in twofold increments (1:2 to 1:32) and tested for LSF activity by the Sindbis virus neutralization system. Undiluted serum lost all LSF activity when heated at 56°C for 30 min. The enhancing effect of LSF on neutralization decreased as serum concentration decreased (Table 1). Gradual loss of LSF effect was demonstrated in both sera between 1:4 and 1:32 dilutions. Similar results were obtained in other tests with human plasma rather than serum.

Stability of LSF at various temperatures. Human serum diluted 1:8 was heated in a water bath at 56°C to determine the effect of LSF. Serum samples were removed after 5, 10, 15, 20, 25, 30, 45, and 60 min of heating and used as Sindbis virus diluent in the neutralization

| Human serum no. | Serum dilution | Treatment | LNI* |
|-----------------|----------------|-----------|------|
| SP-23           | Undiluted      | Unheated  | 4.2  |
| SP-23           | Undiluted      | Heated at | 2.4  |
|                 |                | 56°C (30 min) |     |
| SP-23           | 1:2            | Unheated  | 4.2  |
| SP-23           | 1:4            | Unheated  | 4.1  |
| SP-23           | 1:8            | Unheated  | 3.6  |
| SP-23           | 1:16           | Unheated  | 3.0  |
| SP-23           | 1:32           | Unheated  | 2.5  |
| SP-24           | Undiluted      | Unheated  | 3.8  |
| SP-24           | Undiluted      | Heated at | 2.8  |
|                 |                | 56°C (30 min) |     |
| SP-24           | 1:2            | Unheated  | 3.9  |
| SP-24           | 1:4            | Unheated  | 3.8  |
| SP-24           | 1:8            | Unheated  | 3.3  |
| SP-24           | 1:16           | Unheated  | 2.9  |
| SP-24           | 1:32           | Unheated  | 2.4  |

* Log 10 neutralization index for Sindbis virus.

| Time of storage (days) | Level of LSF* at various temp |
|-----------------------|--------------------------------|
|                       | 24°C | 4°C | -20°C | -56°C |
| 3                     | 1.2  | 0.8 | 1.6   | 1.2   |
| 8                     | 0.9  | 1.3 | 1.5   | 1.3   |
| 15                    | 0.2  | 1.0 | 0.9   | 0.9   |
| 22                    | 0.3  | 0.9 | 1.1   | 1.2   |
| 57                    | ND†  | 0.5 | 1.1   | 1.0   |

* LNI in unheated serum minus LNI in heated serum (LNI = \(\log_{10}\) neutralization index for Sindbis virus).
† Not done.
TABLE 3. Effect of human labile serum factor on neutralization of five arboviruses

| Virus       | Treatment of virus diluent | Virus titer (log_{10} PFU/ml) | LNI\(^c\) | ΔLNI\(^d\) |
|-------------|---------------------------|--------------------------------|----------|----------|
|             |                           | Virus + normal serum\(^a\) | Virus + immune serum\(^b\) |           |          |
| Sindbis     | Unheated                  | 9.6                            | 5.9      | 3.7      | 1.3      |
| Sindbis     | Heated at 56°C (30 min)   | 9.3                            | 6.9      | 2.4      | 1.0      |
| VEE         | Unheated                  | 8.1                            | 5.7      | 2.4      | 1.0      |
| VEE         | Heated at 56°C (30 min)   | 8.0                            | 6.6      | 1.4      | 0.6      |
| EEE         | Unheated                  | 11.2                           | 7.0      | 4.2      | 1.6      |
| EEE         | Heated at 56°C (30 min)   | 10.9                           | 8.3      | 2.6      | 1.5      |
| WEE         | Unheated                  | 10.3                           | 6.3      | 4.0      | 1.5      |
| WEE         | Heated at 56°C (30 min)   | 10.4                           | 7.9      | 2.5      | 1.5      |
| SLE         | Unheated                  | 7.9                            | <3.3     | >4.6     | >1.4     |
| SLE         | Heated at 56°C (30 min)   | 8.4                            | 5.2      | 3.2      |          |

\(^a\) Heated normal rabbit serum diluted 1:10.
\(^b\) Heated immune rabbit serum diluted 1:10.
\(^c\) Log_{10} neutralization index.
\(^d\) LNI in unheated serum minus LNI in heated serum.

test. Complete loss of LSF occurred when serum was heated for only 5 min.

Samples of human serum diluted 1:8 were stored at 24, 4, −20, and −56°C for 57 days and tested periodically to determine the stability of LSF at these temperatures. As shown in Table 2, the neutralization-enhancing effect of LSF was still present in serum stored at 24°C for 8 days but was absent at 15 days. Serum stored at 4°C for 22 days enhanced Sindbis neutralization; however, after 57 days the effect was considerably diminished. Serum stored at −20 and −56°C for 57 days showed no loss in LSF activity. One serum, not tabulated, retained LSF activity for at least 204 days while stored at −56°C.

Effect of freezing and thawing. Human serum diluted 1:8 in HBSS and frozen and thawed 10 times revealed no loss in LSF activity as a result of this treatment. A gradual decrease in pH occurred during the freeze-thaw cycles but had no apparent effect on the neutralization results.

Effect of LSF on neutralization activity of immune sera from different species. Human serum diluted 1:8 was used as virus diluent in Sindbis neutralization tests with immune rabbit serum and immune mouse ascitic fluid. Since the neutralization activity of both of these immune sera was equally enhanced, these results suggest a lack of species specificity of LSF.

Effect of LSF on neutralization of several different arboviruses. Neutralization tests were carried out with VEE, EEE, WEE, and SLE viruses by using immune rabbit sera and normal rabbit serum. Sindbis virus neutralization was done simultaneously to provide a base line of LSF activity. Virus dilutions were made in normal human serum that had been stored at −56°C for 204 days and diluted 1:8. The results (Table 3) indicate enhanced neutralization of all five arboviruses.

Effect of guinea pig complement on Sindbis neutralization. Lyophilized guinea pig complement was reconstituted and tested at dilutions of 1:5, 1:50, and 1:500 to determine its effect on neutralization of Sindbis virus by immune rabbit serum, as compared with undiluted human plasma. There was little if any enhancement of neutralization by the complement.

The same concentrations of guinea pig complement were mixed with heat-inactivated human plasma to determine whether it would restore the lost LSF activity of the plasma. No restoration of neutralization-enhancing activity was observed.

Effect of dialysis on LSF. Undiluted human serum was dialyzed overnight and then diluted 1:8 and tested for LSF activity by the Sindbis neutralization system. Dialyzed and undialyzed sera enhanced neutralization of Sindbis virus equally.

DISCUSSION

Some laboratories use normal serum routinely in virus neutralization tests as a source of LSF, whereas other laboratories almost never use normal serum. Whether normal serum is used is often determined by the availability of suitable serum; it must be free from neutralizing antibodies and nonspecific inhibitors of the viruses being tested. Furthermore, it must contain detectable LSF.

As pointed out by Allen et al. (2) in their re-
view of viral inhibitors in normal sera, many
geriatric investigators routinely heat-inactivate serum
specimens before testing them for neutralizing
antibody, but others do not. It is well established
that serum must be heat-inactivated for use in
the complement fixation test; however, the neces-
sity for heat-inactivating serum before testing for
virus-neutralizing antibodies is not uniformly rec-
ognized. The reasons for heating are several: to
remove nonspecific viral inhibitors, to inactivate
infectious virus in serum collected during viremia,
and to inactivate labile components that may
irregularly enhance virus neutralization. Con-
cerning the latter, improper storage of paired
diagnostic specimens before testing may cause
loss of natural LSF from the first specimen, but
not the second, and may cause a false-positive
result.

Hammon and Work (9) discussed the im-
portance of LSF in sera being tested for arbo-
virus-neutralizing antibodies. They pointed out
the necessity of LSF to sera that have been
stored under various conditions before being
tested. For uniformity, there appears to be a
definite advantage in heat-inactivating such sera
before adding LSF.

Obtaining large volumes of normal serum
often is difficult, and checking the serum to
confirm usable LSF activity and absence of con-
flicting antibodies is time consuming; it is, there-
fore, a matter of simple economy to know how
much the serum can be diluted and still retain
LSF activity. Our studies indicated no loss in
activity of normal human serum at a 1:4 dilu-
tion and very little at 1:8. Certainly, use of a
1:5 dilution rather than undiluted serum ap-
ppears warranted. These findings are in accord
with those of others (9) who have reported that LSF
gains in lost effectiveness between dilutions of
1:5 and 1:10. We have found in concurrent
studies that human plasma can be diluted up to
1:10 without detectable loss in LSF activity.
Although human plasma can be used in place of
serum as a source of LSF, some problems are
encountered; plasma clot formation may occur
during serum-virus incubation. Although these
clots do not appear to alter neutralization en-
hancement, they occasionally disrupt cell mono-
layers in plaque assay bottles.

The term “fresh serum,” as used by investiga-
tors in discussing its effect on virus neutraliza-
tion, has been undefined in most reports. It
is important to know how to store normal serum
for maximum retention of LSF activity. The results
of our studies indicate no loss in activity at either
-20 or -56 C for at least 2 months; in fact, at
the latter temperature no loss was noted for 204
days. Since no appreciable loss in activity oc-
curred during 8 days at room temperature and
during 22 days at 4 C, the necessity for rapid
processing of normal serum for freezing is not as
great as previously assumed. This allows for a
more relaxed scheduling of laboratory work
load. It is also apparent that serum to be used
as an accessory factor can be frozen in bulk if
desired, since freezing and thawing for at least
10 times have been shown not to alter LSF ac-
tivity.

The common practice is to heat-inactivate
serum specimens at 56 C for 30 min to level off
LSF activity. It appears that this time period is
excessive, since, in our tests, heating at this
temperature for 5 min was as effective as 1 hr.

Our studies did not include immune sera of
enough animal species to prove lack of specificity
of normal serum for LSF activity; however, iden-
tical results were obtained when human serum
LSF was used with immune rabbit serum and with
immune mouse ascitic fluid. These are the two
most frequently used antibody preparations in
arbovirus laboratories.

Enhancement of neutralization by LSF was
clearly demonstrated for five different arboviruses.
These results confirm earlier reports on enhanced
neutralization of EEE (7) and WEE (4, 5, 16, 22)
viruses by normal fresh serum.

Several investigators have shown that fresh
guinea pig serum enhances virus neutralization
(15, 17, 19); others have been unable to demon-
strate such enhancement (6, 11). It has been sug-
gested that complement is the labile component
responsible for enhancement of neutralization
(4, 15, 16). However, Howitt (12) found that fresh
guinea pig complement had no effect on the neutralization of WEE virus by immune
horse serum. Our results are in accord with those
of Howitt since complement did not restore lost
LSF activity to heated human plasma.

Although we do not know the chemical nature
of LSF, its ability to pass a cellophane mem-
brane is indicative of a molecular weight of
>25,000 (3).

At the beginning of this study, we assumed
that any human serum free from antibodies to the
viruses of interest would be a satisfactory source
of LSF if stored properly. This assumption is not
necessarily correct. No LSF could be detected
in two of the serum specimens tested in our
study, despite the fact that these two specimens
were from a donor whose serum several months
earlier had exhibited LSF activity. This finding
indicates the advisability of pretesting all sera
used as a source of LSF for LSF activity as well
as for freedom from specific antibodies. The
absence of LSF in some sera may account for
confusing reports concerning the enhancing
effect of fresh serum.
LABILE SERUM FACTOR

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