A New Method to Detect Lymphocytic Choriomeningitis Virus-Specific Antibody in Human Sera

By F. LEHMANN-GRUBE AND J. AMBRASSAT

Heinrich-Pette-Institut für experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany

(Accepted 27 April 1977)

SUMMARY

A plaque reduction method for measuring lymphocytic choriomeningitis virus-sensitizing antibody in human serum is described. One volume of virus and one volume of serially diluted human serum were mixed and incubated for 2 h at 37 °C. One volume of suitably diluted anti-human immune globulin antiserum was added and incubation continued for 0.5 h. Residual infectivity was then determined by means of a plaque assay employing L cell monolayer cultures and a methyl cellulose containing overlay medium. Of 75 sera from as many persons, 20 were positive with titres ranging from 640 to 10240. All positive sera were from verified cases or from persons who had had occupational contact with the virus. Close correlation of results was found between this method and a neutralization test employing mice, the one exception indicating that the in vitro assay for sensitizing antibody is more sensitive than the mouse assay for neutralizing antibody.

INTRODUCTION

Although the lymphocytic choriomeningitis (LCM) virus has been known for more than 40 years to be pathogenic for man (Armstrong & Dickens, 1935; Rivers & Scott, 1936), the methods at present available to detect human infections cannot be considered satisfactory. It is relatively easy to diagnose the acute disease by isolating the virus and/or demonstrating a rise of virus-specific antibody. Usually, however, the LCM virus is not suspected immediately after onset, and the laboratory may be called upon months or even years later to make a diagnosis. At this time attempts at virus isolations are futile and most standard procedures to detect antibody, such as complement fixation and neutralization tests as well as immunofluorescent methods, fail to give reliable information, the main difficulty being that spurious negative results are often obtained (Lehmann-Grube, 1971; Hotchin & Kinch, 1975). The only procedure we have found over the years which allows a valid diagnosis to be made in retrospect, even decades after the acute infection, has been determination of the neutralizing index in mice (Lehmann-Grube et al. 1960; Scheid et al. 1960). This test, however, has severe limitations. In particular, it is based on animals, requires a considerable amount of serum, and is prone to non-specific reactions.

In this report we describe how LCM virus-specific antibody can be quantitatively determined by its ability to reduce the number of virus plaques in a monolayer, provided its neutralizing potential is enhanced by the addition of antibody directed against human immune globulin. Though this assay measures sensitizing rather than neutralizing antibody, it is specific and sensitive. Furthermore, it is easy to perform and appears to be a useful
new procedure to determine infections with LCM virus which have occurred recently as well as up to 30 years ago.

METHODS

Cell cultures. Growth medium for L cells, clone 929 (Sanford, Earle & Likely, 1948/49), consisted of minimum essential medium (MEM; Eagle, 1959) supplemented with non-essential amino acids (Lockhart & Eagle, 1959) and 5% heat-inactivated ordinary calf serum. The pH was maintained at 7.2 with NaHCO₃, the concentration of which depended on the experimental conditions; under 5% CO₂ at 37 °C it was 0.14%.

Virus and virus assay. Strain WE was used (Rivers & Scott, 1936). Because of the great thermal lability of LCM virus, small samples in ampoules were snap-frozen before storage at −70 °C. Also, unless stated otherwise, manipulations involving virus were always performed at ≤ 4 °C and all diluents contained heat-inactivated calf serum.

A plaque assay suitable for our purpose has been developed by Mircea Popescu from this laboratory (to be published). Its essential feature is that cells are maintained under conditions which allow their rapid multiplication for at least 1 day after infection. This is achieved by using cultures with incomplete monolayers and which is almost fluid, rather than the commonly employed semi-solid overlay medium. To facilitate counting of plaques, a highly cytolytic variant of LCM virus was used (Popescu & Lehmann-Grube, 1976). Plastic Petri dishes, 3.5 cm in diam. (‘FB-6-TC’, Linbro, Hamden, Conn., U.S.A.), were seeded each with 3 × 10⁵ L cells in 2 ml of growth medium. After incubation at 37 °C under 5% CO₂ for not longer than 24 h the media were withdrawn and virus in volumes of 0.1 ml of reaction medium (see below) was allowed to adsorb for 45 min at 37 °C under 5% CO₂. The cells were then covered with 2 ml of growth medium containing methyl cellulose, 400 cP, at a final concentration of 0.9%. After 3 days of incubation at 37 °C in a 5% CO₂ incubator, 1.5 ml of medium without methyl cellulose was spread on top of the overlay medium and incubation was continued for another day. On the 5th day the plates with all their contents were immersed in a fluid consisting of 0.2% crystal violet in 10% formaldehyde. After a few minutes the plates were rinsed with water and dried to be evaluated when convenient.

In vitro assay for measuring LCM virus-sensitizing antibody. Optimal conditions have been determined experimentally (Ambrassat, 1977). The procedure finally adopted was as follows. For the production of anti-human immune globulin (Ig) antiserum, human immune globulin (‘Beriglobin’, Behringwerke, Marburg/Lahn, Federal Republic of Germany) at a concentration of 10 mg in 1 ml of Hanks’ balanced salt solution (BSS; Hanks & Wallace, 1949) was emulsified with the same volume of Freund’s complete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) and 1 ml of this material was inoculated intramuscularly into four sites on a rabbit. Four and 6 weeks later the animal received 5 mg of globulin together with Al(OH)₃. Blood was taken after 2 more weeks. Booster inoculations of globulin together with aluminium hydroxide and bleedings 2 weeks thereafter were repeated at intervals. Reaction medium consisted of MEM with 0.056% NaHCO₃ (final concentration), 0.01 M-tris (hydroxymethyl)amino methane (tris), 5% heated calf serum, and penicillin and streptomycin, 100 units and 100 µg/ml, respectively. Virus-sensitizing antibody was measured as follows. Sera were diluted 10-fold with reaction medium and heated for 30 min at 56 °C. They were further diluted in twofold steps up to 1:5120. Virus was diluted with reaction medium so as to contain 3000 plaque-forming units (p.f.u.)/ml. To 0.5 ml of each serum dilution 0.5 ml of virus dilution was added. Tubes were tightly stoppered and incubated in a water bath at 37 °C for 2 h. Heat-inactivated anti-human Ig
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antiserum was diluted 3·3-fold with reaction medium and 0·5 ml was added to each tube which now contained virus (1000 p.f.u./ml), serially diluted human serum and antiserum against human gamma globulin (diluted 1:10). Incubation at 37 °C was continued for 30 min, after which time 0·1 ml of the mixture (containing, in theory, 100 p.f.u.) was spread on each of two L cell monolayer cultures. The plaque assay was then performed as described above. Suitable controls were included.

Mouse assay for measuring LCM virus-neutralizing antibody. The method by which the neutralizing index (NI) of a human serum was determined has been described (Lehmann-Grube et al. 1960). With a few modifications, introduced by R. Ackermann and his colleagues (R. Ackermann, personal communication), it was performed as follows. Diluent consisted of a tris-buffered balanced salt solution (dissolved in 1000 ml of water were tris, 3·60 g; NaCl, 9·00 g; KCl, 0·42 g; MgCl₂·6H₂O, 0·20 g; CaCl₂·2H₂O, 0·24 g; glucose, 1·00 g; phenol red, 0·01 g; pH adjusted to 7·5 with 1 N-HCl) plus 10 % heat-inactivated calf serum and penicillin and streptomycin at final concentrations of 100 units and 100 μg/ml, respectively. For the preparation of virus, mice (colonized NMRI mice, Zentralinstitut für Versuchstierzucht, Hannover, Federal Republic of Germany) were infected intracerebrally when less than 2 days old. Six days later brains were processed with diluent into a 10⁻⁹ homogenate which was centrifuged in the cold at approx. 2500 g for 10 min. The supernatant was stored in samples at −70 °C. For the test, virus was diluted serially 10-fold. Equal volumes of virus and undiluted, non-inactivated (!) serum were mixed and held for 2 h at 37 °C. Five mice were inoculated intracerebrally with each mixture, each animal receiving 0·03 ml, and calculation of the titre (Fazekas de St Groth, 1955) was based on mortality between the 5th and the 14th day (LD₅₀). A control titration (late control) was done under similar conditions except that diluent instead of serum was added to the virus, and the accuracy was raised by using 10 mice per 10-fold dilution step. To monitor loss of infectious virus during incubation an early control was included. The NI is the log₁₀ quotient of the virus titres determined in the absence (late control) and in the presence of human serum. Most human sera contain virus-inactivating substances of unknown nature (Scheid et al. 1960). These normal constituents have been found to reduce virus titres by up to log₁₀ 2·0. Thus, a NI is considered significant only if it is higher than 2·0.

RESULTS

Initial attempts to titrate neutralizing antibody by means of a conventional plaque reduction assay have failed. If heat-inactivated, most sera from verified cases exerted little or no effect on plaque numbers. When the sera were employed in their native state, they significantly reduced plaque numbers at dilutions as high as 1:40, whether they came from persons who had had LCM or not.

With the new assay, which detects sensitizing rather than neutralizing antibody, individual sera from 75 persons were studied. Ten came from former patients who had had clinical lymphocytic choriomeningitis, most often in the form of non-bacterial meningitis, up to 30 years ago. In each case the etiology had been confirmed by isolation of the agent and/or demonstration of a rise of LCM virus-specific antibody. A group of 18 sera came from research workers and laboratory technicians who had had occupational contact with LCM virus for periods up to 6 years. A third group was obtained from 47 regular blood donors whose previous history, either clinically or epidemiologically, was unknown to us.

The tests were performed by one person who received the sera coded without knowing their origin. The results are depicted in Fig. 1. Two patterns of response were observed. The
Fig. 1. LCM virus-sensitizing antibody in 75 sera from 75 persons. (A number of sera were diluted 1:40 to 1:640 in a first test and, only if positive, up to 1:10240 in a repeat test. This explains why there are fewer data points at dilutions 1:1280 and above.)

Fig. 2. Further reduction of LCM virus plaque numbers at lower dilutions of human immune sera caused by increase of the concentration of anti-human immune globulin antibody in the reaction mixture. Results obtained with two different sera are represented by circles and squares. ○ --- ○, □ --- □, final dilution of anti-Ig antiserum 1:10; ● --- ●, ■ --- ■, final dilution of anti-Ig antiserum 1:5.
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Table 1. Results of search for LCM virus-sensitizing antibody in 75 sera from 75 persons

<table>
<thead>
<tr>
<th>Sera from</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persons with verified LCM disease in the past</td>
<td>10</td>
</tr>
<tr>
<td>Persons having had occupational contact with LCM virus</td>
<td>18</td>
</tr>
<tr>
<td>Persons not known to have had LCM disease or virus contact</td>
<td>47</td>
</tr>
</tbody>
</table>

Table 2. LCM virus-specific antibodies in human sera determined in parallel with mouse test (neutralizing antibody) and with cell culture plaque test (sensitizing antibody)

<table>
<thead>
<tr>
<th>Result</th>
<th>No. of sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive with both methods</td>
<td>19</td>
</tr>
<tr>
<td>Positive only with mouse test</td>
<td>0</td>
</tr>
<tr>
<td>Positive only with plaque test</td>
<td>1</td>
</tr>
<tr>
<td>Negative with both methods</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
</tr>
</tbody>
</table>

The majority of sera did not significantly affect plaque numbers. In contrast, 20 sera exerted inhibitory effects which, in most cases but not always, reduced plaque numbers to zero. Reduction was most marked at dilutions 1:160 and 1:320. When sera were used at higher concentrations, more plaques were counted. Their numbers decreased when the reaction mixture contained more antibody against human immune globulin (Fig. 2). Further observations, not immediately apparent from Fig. 1, may be summarized as follows. There was no exception to the rule that a serum reducing plaque numbers at dilution 1:160 did so at higher dilutions. A few sera were still markedly active when diluted 1:10240. On the other hand, sera not suppressing plaques at dilutions 1:640 had no effect when tested at dilutions 1:40.

Further evaluation was based on the following definitions. Titre is the reciprocal of that serum dilution (before addition of anti-human Ig antiserum) which is closest to reducing plaque numbers by 50%, and a serum is considered positive when its titre is 160 or higher. On this basis the titration results were considered in relation to the previous history of the persons from whom the sera had been obtained (Table 1). While there was good agreement between observed and expected results in two groups, the proportion of positive sera in people who had handled the virus was surprisingly high. To confirm the specificity of these results, all 75 sera were also tested for neutralizing antibody by means of the mouse assay (Table 2). With one exception there was agreement of the results based on sensitizing antibody determined in vitro and neutralizing antibody determined in vivo. The one aberrant serum had a NI of 1.4, which could not be considered as significant. It came from a person known to have had lymphocytic choriomeningitis 19 years ago. The high NI of this person’s serum soon after the illness had decreased over the years (R. Ackermann, personal communication). The titre of this serum was also relatively low when measured as sensitizing antibody (640). It should be stressed, however, that antibody titres determined both in vitro and in vivo were not in any way correlated with the time periods that had elapsed between disease and taking the serum.
DISCUSSION

Not long ago the lymphocytic choriomeningitis (LCM) virus was considered to be of
minor importance for man, the principal reservoir in nature being persistently-infected grey
house mice (Mus musculus; Lehmann-Grube, 1971). It now appears that the relevance of
this agent might have been underestimated. Numerous cases have become known which
prove that the golden hamster (Mesocricetus auratus), a laboratory animal as well as
a favourite family pet in many parts of the world, may transmit LCM virus and thus cause
disease to its holder (Lewis et al. 1965; Armstrong et al. 1969; Ackermann et al. 1972; Hirsch
et al. 1974; Hotchin et al. 1974; Deibel et al. 1975; Gregg, 1975). Even more important
appears to be the claim that the LCM virus is capable of causing malformations by infecting
the embryo via the pregnant mother (Ackermann et al. 1974; Sheinbergas, 1976). Conse-
quently, the number of requests to virus laboratories to include the LCM virus in a search
for the etiological agent in human diseases has recently risen sharply.

The lack of an assay that is reliable and at the same time easy to execute has necessitated
the development of new methods for the measurement of LCM virus-specific antibody.
A microplaque reduction assay described by Hotchin & Kinch (1975) appears to be easy to
perform but is not as reliable as hoped. According to the authors' own account, only 47 of
55 human infections with LCM virus were verified by this method. Our efforts initially
aimed at developing a conventional plaque reduction assay for demonstrating human anti-
body to LCM virus. Experiments in which human sera were directly tested for the effect
they had on plaque titres were disappointing. The results were dramatically improved by
adding antiserum against human immune globulin to the reaction mixture. Originally
introduced by Notkins and co-workers (1966), this procedure has demonstrated in numerous
instances that antibody may be attached to a virion without affecting its infectivity.
Antibody titres thus obtained ranged from 640 to 10240. Maximal reduction of plaque
numbers occurred at dilutions 1:160 and 1:320. When sera were used at higher concen-
trations, more plaques were counted. The conclusion that this paradoxical increase
represented a zone phenomenon caused by insufficient concentrations of anti-human Ig
antibody relative to the large numbers of immune globulin molecules present in serum at
low dilutions was confirmed by the results of an experiment which showed that increasing
the concentration of antibody against human immune globulin reduced the magnitude of
the zone phenomenon. The same experiment revealed that more rabbit anti-human Ig
antiserum in the reaction mixture resulted in higher plaque counts at higher dilutions of
human sera. We find it difficult to interpret this phenomenon; possibly the higher con-
centration of protein exerted a protective effect on this very labile virus.

Possible explanations for the mechanism underlying virus sensitization and neutralization
by anti-antibody have been discussed by Notkins (1971). For the present purpose we are
content with saying that the titration results were as expected, thus indicating that the
interaction is specific. In 10 sera from as many former patients, sensitizing antibody was
demonstrated. Of the 47 sera expected to be negative (or positive in only a low proportion)
one contained LCM virus-specific antibody. Of 18 sera from persons working or having
worked with this virus 10 were positive. This frequency of laboratory infections was quite
unexpected because none of the persons with virus-specific antibody had had an illness
resembling lymphocytic choriomeningitis. It is generally assumed that inapparent infections
with LCM virus are rare (Lehmann-Grube, 1971). Presumably, our laboratory strains are
attenuated for man. To obtain further evidence for the specificity of the new procedure, all
75 sera were also tested for neutralizing antibody with the mouse assay which, with all its
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inadequacies, is nonetheless quite reliable. There was good correspondence of results, the one exception indicating that the plaque reduction assay may be more sensitive than the mouse assay.

We sincerely thank Susan Mählitz and Edeltraud Bugislaus for their excellent technical assistance. The 47 sera from persons not known to have had LCM disease or virus contact were kindly supplied by Dr B. von Eisenhart-Rothe, Abteilung für Transfusionsmedizin, Chirurgische Universitätsklinik, Hamburg, and 10 other sera analysed in this study were given to us by Professor R. Ackermann, Universitäts-Nervenklinik, Köln. This work was financially supported by the Bundesministerium für Jugend, Familie und Gesundheit, Bonn.

REFERENCES


*(Received 21 March 1977)*