THE PROVOCATION OF LATENT LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTIONS IN MICE BY TREATMENT WITH ANTILYMPHOCYTIC SERUM

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Antilymphocytic serum (ALS) is now known to have a pronounced immunosuppressive effect on animals into which it is injected (1–4). In certain experiments during prolonged treatment with ALS in dogs and monkeys some animals have died with symptoms of acute viral infections (3, 5). It has been suggested (6) that these infections were caused by a provocation of occult viruses brought about by abolition of an antiviral immunity in the hosts. However, direct experimental data supporting such a theory have hitherto been lacking.

The question of whether it is possible to provoke latent virus infections by a suppression of the immune mechanism of the host obviously has both theoretical and practical implications. It would therefore seem necessary to investigate the matter. The mouse-lymphocytic choriomeningitis (LCM) virus system is ideal for this purpose. After recovery from an LCM virus infection of adult mice the virus often persists in the mice for a long time, probably for the rest of the life of the animals (7).[1] The mice are perfectly healthy, have moderate high titers of complement-fixing antibodies, and are immune against reinfection. In spite of this, trace amounts of virus may occasionally be found in the blood and moreover in certain organs, such as the kidneys, the presence of virus can be demonstrated regularly a year or more after the infection. A perfect balance between the LCM virus and its host seems therefore to have been established. It is the purpose of this report to present data which show that prolonged treatment with ALS can tip such a balance in favor of the virus and provoke a viremia with high titers of virus. No correlation between the titers of complement-fixing antibodies and the blood virus titers has, however, been demonstrated.

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[1] Larsen, J. H. 1967. Personal communication.

Materials and Methods

Animals.—The mice were highly inbred AKR mice raised at our institute. The rabbits used for the preparation of antilymphocytic serum and control sera were noninbred grey and white rabbits obtained commercially.

Antilymphocytic serum was prepared from rabbits by a modification of the method described by Gray et al. (8) and the procedure used is described in detail by Lundstedt and Volkert (9). The cell suspensions used for immunization were a mixture of lymph nodes and spleen cells from normal female mice. 25×10^6 cells of this mixture in a volume of 0.1 ml emulsified with an equal volume of complete Freunds adjuvant were injected into each of the footpads of the recipients. 4 wk later booster injections of 100×10^6 lymph node and spleen cells without adjuvant were given intraperitoneally on 3 consecutive days, and 1 wk after the last injection the rabbits were bled. The serum was collected and stored at −20°C after addition of merthiolate 1:10,000. The rabbits were bled several times but booster injections of 100×10^6 cells were always given 1 wk prior to bleeding. The serum prepared in this way is strictly speaking an antiserum against all the different cells in the preparation, but for the sake of simplicity it will be referred to as antilymphocytic serum (ALS).

Control sera were sera from normal rabbits stored as described for the ALS.

Potency tests of the ALS were carried out by in vitro cytotoxic tests and by determining the effect in vivo on the number of the blood lymphocytes. The cytotoxic tests were performed according to the method described by Gorer and O'Gorman (10). Routine tests gave cytotoxic indices of approximately 0.25 with cell concentrations of 10×10^6 cells and 0.25 ml of ALS injected intraperitoneally into 20-g mice caused a fall in the cell count to approximately one third of the original values within 150 min.

LCM virus strain originated from Dr. Traub, Germany, and has been used in this laboratory for many years (11). The virus was passed when necessary by intraperitoneal inoculation of mice. The virus preparations consisted of a 10% spleen suspension from infected mice and was stored at −70°C. The virus titrations were carried out as previously described (12). Ordinary white Swiss mice were used for the test. The end points were calculated in accordance with the method of Kärber (13) and expressed as log 10 LD_{50}/0.03 ml. When only one of the inoculated mice died after the 7th day of the observation period the titer is expressed as trace. Blood virus titers were determined on whole blood taken from the extraocular venous plexus.

Complement-fixing antibodies were titrated as described previously (14). The method was altered only in that the antigen used was prepared from spleens of tolerant virus-carrier mice. The end points of the titrations were the last tubes showing less than 50% hemolysis. The sera were stored at 4°C and the tests were carried out once a week.

EXPERIMENTAL

Mice which have become immune to the LCM virus by either a natural infection or by an artificial infection have been employed. The natural infection was brought about in mothers nursing infected babies. For the purpose of studying tolerance to the virus in this laboratory newborn babies are routinely injected with the LCM virus. The babies treated in this way develop a tolerant virus infection, excrete virus in great amounts, and contaminate their mothers (11). This contamination has never been seen to cause an overt disease in the mothers. However, at the time they are separated from their offspring, usually 6–8 wk after the babies are born, they have developed a strong immunity to the virus. They are highly resistant to reinfections and the blood contains high titers of complement-fixing antibodies. Artificial infections are also routinely used in this laboratory in order to produce immunization. This is achieved by intraperitoneal injections of sublethal doses of virus into adult female mice. 4 wk after such injections these mice have also developed a strong immunity to the virus and have

high complement-fixing antibody titers. In both groups the virus has a tendency to persist as described in the introduction. However, no spontaneous provocation of the virus has ever been observed.

Experiment I.—

In the first series of experiments 24 mothers of infected babies were employed. All had nursed their babies for 7–8 wk and were used for the experiment within a week after the babies had been removed. The blood of each mouse was tested individually for virus in dilutions of 10^1–10^4 and for complement-fixing antibodies in dilutions of 8–1024. The mice were then divided at random into three groups of eight mice each.

In group A all mice received 0.25 ml of ALS intraperitoneally six times a week for 7 wk. On days 10, 20, 27, 34, 42, 53, and 74 individual blood samples were tested for virus and complement-fixing antibodies in the dilutions mentioned above. The first 3 wk of treatment were well tolerated, but from then on all mice began to look ill and two died between the 34th and 42th day. After the ALS injections were discontinued on the 44th day of the experiment, the remaining mice recovered slowly.

Groups B and C comprised the control groups. The mice in group B received normal rabbit serum intraperitoneally in amounts of 0.25 ml following the same schedule as described for group A. The treatment was well tolerated throughout the experiment. The mice in group C did not receive any treatment. Blood tests for virus and complement-fixing antibodies were performed individually on each mouse in both groups on the same days as the mice in group A were tested.

The virus titration results for all three groups are given in Table I. Where those mice that received ALS are concerned it is seen that none of them had detectable virus in their blood at the beginning of the experiment. On day 10 and day 20 after the ALS injections were initiated, occasionally mice could be found with trace amounts of virus in the blood but as it is apparent from the control group C this is not more than is to be found in untreated immune mice. However, during the last part of the ALS treatment the situation changed and all mice developed a clear-cut viremia with virus titers varying from 1.3 to 3.5. In none of the mice was the viremia detected earlier than 27 days after the serum injections were started, and in some it was even later. In two mice the viremia was detected in only one blood sample. In four mice the viremia lasted until the ALS treatment was discontinued and then disappeared within 10 days. One mouse, the one with the highest blood virus titer, had a more prolonged viremia than the others, but even in this case practically all the virus in the blood had disappeared when the last test was done. As mentioned earlier two of the mice had died during the experiment and we only know that viremia developed.

In both control groups B and C trace amounts of virus could be found in only two mice during the whole experimental period; in none of the others was virus detectable in the blood.

Where the complement-fixing antibodies are concerned it is apparent from Table II that in untreated mice the titers at the beginning of the experiments varied about eightfold from mouse to mouse and about half of the animals had

TABLE I

LCM Virus Titers in the Blood of Mice Immunized by a Natural Infection and Subsequently Treated with ALS

Animal groups	Blood virus titers							
	days....0	10	20	27	34	42	53	74
	ALS treatment							
Experimental group A receiving ALS								
Mouse 1	n. tr.	tr.	n. tr.	n. tr.	1.3	n. tr.	n. tr.	n. tr.
" 2	n. tr.	n. tr.	tr.	2.3	2.5	3.0	3.5	tr.
" 3	n. tr.	n. tr.	n. tr.	1.3	n. tr.	tr.	n. tr.	n. tr.
" 4	n. tr.	n. tr.	n. tr.	n. tr.	1.8	—	—	—
" 5	n. tr.	n. tr.	n. tr.	n. tr.	1.0	2.0	n. tr.	n. tr.
" 6	n. tr.	n. tr.	n. tr.	1.3	1.8	2.3	n. tr.	n. tr.
" 7	n. tr.	tr.	n. tr.	1.3	1.8	—	—	—
" 8	n. tr.	tr.	n. tr.	1.0	2.0	2.0	n. tr.	n. tr.
Control group B receiving normal rabbit serum	7/8 n. tr.*	8/8 n. tr.	8/8 n. tr.	7/7 n. tr.	7/7 n. tr.	7/7 n. tr.	6/6 n. tr.	6/6 n. tr.
Control group C untreated	8/8 n. tr.	8/8 n. tr.	8/8 n. tr.	7/8 n. tr.*	8/8 n. tr.	8/8 n. tr.	8/8 n. tr.	8/8 n. tr.

n. tr., no trace of virus; tr., trace of virus; and —, not done because of the death of the mouse.

* Remaining mouse had trace amounts of virus in the blood.

a titer of 128. In some of the mice in group C that did not receive any treatment the antibody titers showed a tendency to decline in the following weeks but no great variations were seen. The results for group B very closely resembled those in group C and are therefore not recorded. The mice in group A, however, showed an antibody pattern that was clearly different from the control groups.

TABLE II

LCM Complement-Fixing Antibody Titers of Mice Immunized by a Natural Infection and Afterwards Treated with ALS

Animal groups	Antibody titers							
	days 0	10	20	27	34	42	53	74
	ALS treatment							
Experimental group A receiving ALS								
Mouse 1	128	128	≧1024	1024	256	512	256	512
" 2	128	32	32	512	512	256	128	256
" 3	64	128	128	128	256	512	256	256
" 4	128	32	16	32	32	—	—	—
" 5	128	64	≧1024	1024	256	—	512	512
" 6	512	128	≧1024	256	512	256	256	512
" 7	64	64	≧1024	1024	256	—	—	—
" 8	128	64	≧1024	2048	512	512	256	512
Control group C untreated								
Mouse 1	256	256	128	512	1024	128	256	512
" 2	64	128	128	128	128	32	64	256
" 3	128	256	64	64	64	32	32	—
" 4	256	512	64	32	128	128	32	64
" 5	128	256	64	64	32	32	16	32
" 6	512	256	256	256	128	—	128	128
" 7	128	64	128	128	64	128	32	64
" 8	128	128	128	128	128	128	64	128

—, not done.

In the beginning of the experiment a depression of the antibody titers was demonstrable in three of the mice whereas the titers seemed to remain constant in the others. On day 20, however, before any viremia could be detected, a steep rise in titers occurred in five of the eight mice. Moreover, an increase in antibody titers also occurred in two other mice, but this increase came later and was less pronounced. After this rise most of the antibody titers remained high for about a week or two and then declined to the original level. One mouse had an antibody pattern which differed from the others. In this mouse the

antibody titer was also depressed in the beginning but, contrary to what happened in the others, the titer never rose again. The mouse, however, could not be followed for a very long period as it died between the 34th and 42nd day of the ALS treatment.

Experiment II.—

In the second series of experiments artificially infected mice were employed. The ages of the mice were between 2½ and 3½ months. All mice were infected with LCM virus by intraperitoneal injections of sublethal doses of virus and then divided at random into three groups of eight mice each (groups D, E, and F). To make certain that an infection had been established, the blood of each mouse in all three groups was tested individually for virus in dilutions of 10^1–10^4 and for complement-fixing antibodies in dilutions of 8–1024 6 days after the virus inoculation. The titration results showed that all 24 mice had viremia with virus titers varying from 1.0–1.8 and none of them had complement-fixing antibodies. To ascertain that an immunity developed 20 and 29 days after the virus inoculation all mice in all groups were tested again. The samples on both these days showed that none of the mice had any detectable virus in their blood and that all of them had complement-fixing antibodies in titers varying from 64 to 256.

When the blood samples had been collected on day 29 after the virus inoculation the ALS experiment was begun.

In the experimental group D all mice were injected intraperitoneally with 0.25 ml of ALS six times a week for 7 wk as described for group A in the foregoing experiment. Blood samples for virus and antibody titrations were collected individually following a schedule which was also identical with the one used for group A.

Groups E and F comprised the control groups. The mice in group E received normal rabbit serum intraperitoneally in the same amounts and on the same days the mice in group D were injected. The mice in group F were left untreated. Blood samples from the mice in both control groups were collected and tested individually for virus and antibodies on the same days as those from group D.

As was the case in group A, the mice in group D which received ALS tolerated the treatment well for about 3 wk, but from then on all of them became ill and two died during the 4th and the 5th wk respectively. Of the remaining six mice, two died after the ALS injections were discontinued, but the other four recovered slowly. The control mice receiving normal serum and those which were left untreated remained healthy throughout the experiment.

The data from the virus titrations are recorded in Table III. It is seen that the blood virus titers of the ALS-treated mice resembled those obtained in Experiment I. During the ALS treatment all mice in group D developed viremia. However, as a whole this viremia developed earlier than in the first experiment. As many as four out of the eight mice had rather high virus titers 10 days after the ALS injections were begun. Moreover five out of the eight mice reached virus titers of 2.8 or higher and one a titer as high as 4.5. When the ALS treatment was discontinued the virus titers declined. In two mice which had developed high titers the viremia lasted during the first 12 days, and even on the 74th day one mouse showed a titer of 1.8.

TABLE III

LCM Virus Titers in the Blood of Mice Immunized by an Artificial Infection and Afterwards Treated with ALS

Animal groups	Blood virus titers							
	days.....0	10	20	27	34	42	53	74
	ALS treatment							
Experimental group D receiving ALS								
Mouse 1	n. tr.	n. tr.	n. tr.	1.3	n. tr.	tr.	tr.	tr.
" 2	n. tr.	n. tr.	tr.	1.3	2.3	2.8	1.8	n. tr.
" 3	n. tr.	n. tr.	n. tr.	1.0	1.8	3.0	2.5	1.8
" 4	n. tr.	1.0	n. tr.	tr.	1.3	2.8	n. tr.	n. tr.
" 5	n. tr.	n. tr.	1.5	2.5	3.5	4.5	—	—
" 6	n. tr.	2.5	3.5	—	—	—	—	—
" 7	n. tr.	1.0	tr.	1.0	n. tr.	—	—	—
" 8	n. tr.	1.5	n. tr.	n. tr.	tr.	n. tr.	n. tr.	—
Control group E receiving normal rabbit serum	7/8 n. tr.*	7/8 n. tr.*	7/8 n. tr.*	7/8 n. tr.*	7/8 n. tr.*	7/8 n. tr.*	7/8 n. tr.*	8/8 n. tr.
Control group F untreated	7/8 n. tr.*	8/8 n. tr.	8/8 n. tr.	7/8 n. tr.*	7/8 n. tr.*	8/8 n. tr.	8/8 n. tr.	8/8 n. tr.

For abbreviations see Table I.

* Remaining mouse had trace amounts of virus in the blood.

From Table III it is also apparent that in the control mice only trace amounts of virus could be demonstrated. In the untreated group this was seen on only three occasions whereas it was a more frequent observation in the group which received normal rabbit serum.

In contrast to what had been observed in the first experiment the ALS treatment in this investigation did not influence the antibody level. Neither a depression nor the subsequent increase in titer could be demonstrated. Throughout the whole experimental period there was no significant difference in the antibody titers obtained in the ALS-treated groups and the control groups. The

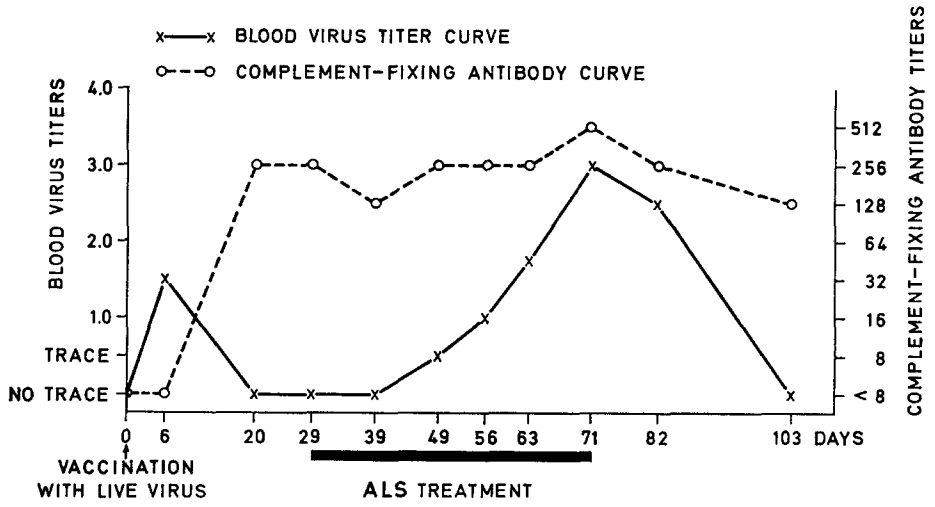

FIG. 1. The effect of ALS treatment on a mouse which had been vaccinated with live LCM virus.

mean titer for all groups remained at a level of about 128 from the beginning to the end of the experiment.

The data from the virus titrations and the antibody titrations obtained in one of the ALS-treated mice are recorded graphically in Fig. 1.

DISCUSSION

The results presented demonstrate clearly that intensive and prolonged ALS treatment of mice can provoke a latent LCM virus infection. Highly inbred mice have been employed in these experiments. Nevertheless, the effect of the ALS on the blood virus titer level varied greatly from mouse to mouse. However, it is noteworthy that in none of the ALS-treated mice did the treatment fail to cause a multiplication of the occult virus. This result was achieved in spite of the fact that all the mice in which it happened were highly immune

to the virus at the time the ALS treatment was begun. In addition to the individual variations there was a clear difference between the results obtained in the two groups of immune mice which received ALS. Taken as a whole the viremia developed earlier and the blood virus titers were higher and remained high for a longer period after the discontinuation of the treatment in the mice employed in the second experiment than in those in the first experiment. The mice used in the two experiments differed at the beginning of the ALS treatment by the way the immunization and the latent virus infection were brought about. In the first experiment the mice employed were mothers of infected babies and these mice had had a natural infection. The mice in the other group were infected artificially by intraperitoneal injection of virus. The antibody levels in the two groups of mice were practically the same at the beginning of the experiment. However, on many previous occasions in this laboratory the immune status of the two kinds of mice have been compared with respect to the immunity which can be conferred to tolerant virus carrier mice by transplantation of lymphoid cells from the immune donors. In such experiments it has repeatedly been found that the potency of the cells from the immune mothers is greater than that of the cells from artificially infected mice (12). Theoretically it seems logical to expect that the animal with the strongest immunity also should be the one with the highest resistance to ALS. This expectation was confirmed by our ALS experiments, provided that one judges the immunity by potency of lymphoid cells and not by the antibody level.

Where the influence on the virus of complement-fixing antibodies is concerned, earlier experimental results from this laboratory (9, 15–17) strongly suggested that these antibodies most probably have no influence on the virus. The data presented in this paper support this view. The results clearly show that the viremia develops in spite of constant and even increasingly high titers of complement-fixing antibodies and the elimination of virus also seems to occur independently of these antibodies. Moreover, the resistance to ALS seems, as pointed out above, to be related to the "cellular" immunity and not to the kind of immunity which can be determined by a measurement of the complement-fixing antibody level. The role of neutralizing antibodies to LCM virus is not yet fully known. However, in this respect it must be pointed out that no one has been able to demonstrate with certainty the presence of these antibodies in either artificially or naturally LCM-infected and immune mice (7, 14, 18–20). The search for another humoral factor, interferon, which might influence the LCM virus has also failed (14). We feel that at present all the available data support the view expressed in an earlier article from this laboratory that a direct cellular activity (a cellular immunity?) plays a very important role in the LCM infection in mice. This hypothesis is also supported by what has been learned about the action of ALS in other experiments. Levey and Medawar (4) have pointed out that ALS apparently inhibits cellular

responses preferentially and it has also been shown that ALS has a very pronounced effect on the "host versus graft" reaction and delayed sensitivity reactions (21)—phenomena which are believed to be entirely cell bound. On the other hand the effect of ALS on a preexisting humoral antibody level is not very strong (2, 22) or, as shown in our experiments, in many cases not demonstrable at all. Very recently one of us (Lundstedt) has obtained some preliminary experimental results in vitro which might indicate that a reaction occurs between LCM immune lymphoid cells and ordinary L cells infected with LCM virus. The interaction between these two types of cells seems to cause the death of the infected cells. It is well known that an in vitro reaction like this occurs between immune lymphoid cells and tumor cells containing new cellular antigens (23–26) and some authors believe that this phenomenon plays an important role in the elimination of malignant cells in vivo (24). If Lundstedt's experiments are confirmed the elimination of LCM virus-infected cells in vivo by the same mechanism would be a tempting hypothesis.

The provocation of latent LCM infections by treatment with ALS strongly suggests that other latent virus infections can be provoked in the same way. The assumption made by other investigators that their animals which died during an ALS treatment were killed by a provocation of occult viruses seems, therefore, very probably correct. In our experiments the virus titers declined again when the injections of ALS were discontinued. However, we know from other experiments (9) that a protracted tolerant LCM infection can be produced in mice when the infection takes place under cover of ALS. Tolerant LCM virus infections might therefore also be the result when latent LCM virus infections are provoked by ALS and the treatment is continued longer than we have done. Such very prolonged ALS treatments are often given in kidney transplantation experiments. As we already have mentioned, the provocation of virulent viruses might mean the immediate death of the subject under treatment. However, one cannot avoid speculating what would happen if the virus which is provoked is a tumor virus and the provocation causes a tolerant infection.

A curious observation in our experiments was that in some mice the ALS treatment temporarily seemed to stimulate the production of complement-fixing antibodies. This happened, however, only in one group of mice. In all groups some variations in antibody titers were noticed, but the rise observed in some of the ALS-treated mice was so large that it can hardly be incidental or due to experimental errors.

The ALS effect on virus infections might have other and important implications for virus research. It might be possible under cover of ALS to cultivate viruses in animals in which these viruses normally do not multiplicate. Some evidence in support of this hypothesis has already been presented by other investigators (27). In spite of the fact that the results obtained hitherto in this respect are only preliminary they are very suggestive.

SUMMARY

The hypothesis that treatment with antilymphocytic serum (ALS) can provoke latent virus infections has been investigated.

In adult mice infections with sublethal doses of LCM virus usually result in the development of immunity to the virus and at the same time to a prolonged latent infection.

In the experiments described an intensive treatment with large doses of ALS was given to mice which had recovered from LCM virus infection. At the beginning of the treatment the mice had high titers of complement-fixing antibodies in their blood and no detectable virus. The data presented show that in spite of the immunity the ALS treatment provoked the occult virus and led to the development of viremia in all the treated mice. In some, very high virus titers were demonstrable. When the ALS treatment was discontinued the viremia disappeard again.

In most of the mice the ALS did not suppress the complement-fixing antibody titers and in some there was even a considerable increase in titer. In such cases the increases in virus titers and in antibody titers were closely related to one another. These results demonstrate once again that the complement-fixing antibodies to the LCM virus in mice probably do not influence the virus.

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