Formation of Antibodies against Salmonella Lipopolysaccharides during a Latent Infection by Friend Murine Leukemia Virus

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SUMMARY

A strain of the Friend leukemia virus (FV6H), after prolonged storage at −60°C, appeared to be attenuated. The progression of Friend disease induced in mice by this virus could be divided into 3 phases: Phase I, a period of about 14 days, during which signs of the disease were limited to the appearance of splenic foci; Phase II, an ensuing phase of 7 additional days, during which moderate splenomegaly and formation of splenic foci occurred, but during which the count of leukocytes in the circulating blood and the blood picture remained within normal limits; and Phase III, the phase of onset and development of typical Friend’s disease.

Coincident with Phases I and II of this disease syndrome, groups of mice showed reduced ability to form circulating antibodies to salmonella lipopolysaccharides. This impaired immunological response was noted following single injections of antigen given 7 or 2 days before or 2 days after the virus and following two injections of antigen 21 days apart when the first injection was given before the virus. The results suggest that significant impairment of immunologic response precedes “clinical” onset of leukemia.

INTRODUCTION

Antibody formation is depressed in mice with established leukemias induced by Friend or Rauscher murine leukemia viruses (2–5, 10, 12, 19, 20, 27, 31, 33). Depression of ability to produce circulating antibody is accompanied by suppression of the appearance of antibody forming cells in the spleen as determined by localized hemolysis in gel (2–4, 10). When high doses of the virus are given, the pathologic characteristics, such as lymphocytosis, monocytosis, and hypertrophy of spleen and liver, ensue almost immediately or at least within a few days (14). Splenic foci are demonstrable within 5 days, together with splenomegaly, with high challenge doses of the Friend virus (6).

Thus, when high doses of these murine leukemia viruses are given, there is virtually no latent period, and the decrease in ability to produce antibody forming cells may reflect the profound disturbances arising from intense splenic erythrocytopenia, invasion of spleen and liver by neoplastic cells, and consequent interruption of function due to impaired circulation.

The established disease induced by the Friend virus may interfere with either the primary or secondary antibody responses (19, 27). Salaman and Wedderburn (27) stated that the injection of virus after the first, but before a second, injection of sheep erythrocytes depressed the secondary response. Odaka et al. (19) reported inhibition of the primary response when virus was given one day after antigen.

The precise course which immunosuppression may follow in Friend virus disease may be complicated. This is especially true of the secondary response which depends on the antigen used, the dose, and the time relationships between primary and secondary stimulation (9, 13, 29). The particular modification of this complex interaction may in turn depend on the point in time or place at which the virus intervenes.

Although earlier work (32) seemed to indicate that immunologic memory does not exist for cells forming IgM, Nossal et al. (18) have clearly shown that IgM memory really does exist and can be elicited by appropriate choice of primary dose, secondary dose, and time interval. The protocol used by us produces a distinct secondary type response, including IgM and IgG antibodies with salmonella antigens (see below).

It seemed desirable to study antibody response during a latent period preceding onset of profound physiologic disturbance for the following reasons: (a) the competitive activity of virus and antigen, which has been the subject of some speculation (3, 5, 27, 31) might be more clearly discernable; (b) the model of preleukemia or early leukemia so established could yield information leading to the development of an early diagnostic test. Diagnosis and early treatment of leukemia during the preleukemic phase might well provide better results than when treatment is begun after the disease becomes well established.

Other models of preleukemia have been studied with respect to antibody-forming ability. So far such studies have been made in animals infected when newborn, either by injection (7, 8, 21) or by vertical transmission from the parent (15). It is believed that murine leukemia viruses under such conditions exert their influence through the thymus gland, which would not be involved in adults.

Although latent periods do not occur in the usual approach to the study of Rauscher and Friend murine leukemias, there is an implication that such preleukemic phases do exist (16).

Several methods for producing long latent periods following injection of the Friend or Rauscher virus are under study. In
this publication, we shall report current results with one of them.

MATERIALS AND METHODS

Virus. The virus (11) used in these studies was obtained from the American Type Culture Collection as a lyophilized suspension. A spleen homogenate prepared in random-bred Swiss mice was stored in a freezer at -60°C in 1963, and it is referred to here as FV6H. In July 1967, an attempt was made to recover the virus from this suspension. Splenomegaly did not occur until about 3 weeks after intravenous injection of this virus pool into random-bred Swiss mice. Such a latent period had not been previously observed (see Results). By sacrificing survivors 11 weeks after intravenous injection of dilutions of the virus in 0.2-ml amounts, and by considering all mice which were either dead or had spleens weighing more than 230 mg (Normal mean + 95% confidence limits), we calculated the ID50 used in these experiments as being 8.3 units.

Mice. These investigations were carried out in random-bred Swiss mice procured from a single commercial dealer (Blue Spruce Farms, Altamont, New York). These animals have always been free of salmonellae as determined by culture and of antibodies against Salmonella typhimurium. They have proved to be far more sensitive to Friend disease than inbred strains such as BALB/c.

Determination of Circulating Antibodies. Antibodies were measured by the very sensitive passive hemagglutination (HA) or hemolysis (HE) technics of Neter et al. (17). Sheep red blood cells (SRBC) preserved in equal volumes of Alsever’s solution under sterile conditions were modified in a solution of S. typhimurium lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Michigan) in phosphate-buffered saline at pH 7.2 (PBS). The LPS was of the type which is extracted from bacterial cells by the Westphal method. Preliminary titration with known positive serum indicated that the optimum concentration for modification of SRBC was 80 μg/mL. The LPS was boiled for one hour before use in the modification procedure. Complement used for HE determinations was obtained from various experiments involving 183 mice, carried out at different times, are collected. After the virus was injected intravenously there was a period of about 3 weeks (Phase I) during which blood leukocyte counts and spleen weights remained within normal limits. During the ensuing 4 weeks (Phase II), blood leukocyte counts and spleen weights increased, leading to the usual pathologic expression of Friend disease (14) by the 7th week (Phase III). As is always the case with this virus-host system, there were great individual variations among animals. Some individuals eventually exhibited grossly enlarged spleens, whereas a few spleens were in the normal range even after the expiration of 7 weeks. The same was true of the leukocyte counts and of the blood pictures. Although erythroblasts occasionally appeared in the blood in large numbers at 3 weeks accompanied by polychromatophilia and anisocytosis in some animals, the appearance of “Friend” cells was always very nearly a terminal event (Table 1). Findings such as these are equally characteristic of random-bred
mice which have been injected with fully active Friend virus preparations, except for differences in the time relationships.

We use the term "attenuated" for virus pool FV6H with some reservations. The results shown in Chart 1 could have been produced simply by a low dose of virus, by a mixture of viable with nonviable virus particles, or by alteration in the nature of the virus as implied by the term.

The FV6H virus produced splenic foci when tested by the method of Pluznik and Sachs (22). Eight days after intravenous injection of the original pool, an average of 4 foci per spleen was counted. After the second passage of virus, 40 foci per spleen were observed. The difference was not statistically significant. The histologic appearance of foci accorded with descriptions given by Metcalf et al. (14) and by Pluznik and Sachs (22).

Hematologic Parameters. The normal peripheral blood leukocyte counts of Swiss Webster mice are subject to wide variations (1, 31). Using tail blood, we determined leukocyte counts on 42 normal mice ranging from 25 to 35 grams in body weight (Table 1).

Leukocyte and mononuclear counts on the blood of mice following injection of FV6H are shown in Table 1. The increase in total leukocytes at 24—35 days seems to correlate well with pathologic changes [polychromatophilia, anisocytosis, erythroblastosis, and the appearance of reticulum cells (or lymphoblasts)] observed in Jenner-Giemsa-stained smears. The marked increase in standard deviation at this time reflects the variation that resulted when some animals exhibited leukocyte counts in the leukemic range while others were still low to high normal values.

Further studies are in progress in the hope of elucidating the nature of this particular virus pool. In the meantime, we have taken advantage of the fairly well-defined latent period of 21 days to study the effect of this physiologic state upon anti-
body production.

**Effect on Antibody Production When Priming Dose Was Administered before the Virus.** The experimental design is shown to the left of the ordinate in Chart 2 (see also Table 2). There was no delay in appearance of antibody in the blood of the virus-infected animals, but the latter fell short in peak response. The impairment evident after the 6th day appeared to become more pronounced as time went on. The infected mice produced some IgG antibody, but, except for a short and very sharp burst of activity at 6 days, the titers were very much lower in the virus-infected animals than in the controls. It should also be noted that most of this activity went on during the stage of preleukemia before onset of the disease.

The fact that priming was done 18 days before the virus was given would lead one to conclude that the impairment in antibody response in this experiment must be interference with the secondary response.

In summary, when priming was done well in advance of virus challenge so that interference with the priming response was not an issue, a moderate interference with the secondary response with respect both to the IgM + IgG and the IgG components was identifiable in the latent period before clinical onset of the disease. This became more prominent as the disease entered the clinical phase (Day 30).

**Effect of “Attenuated” Friend Virus on the Primary Response When Virus Was Administered before Immunization.** After a single large dose of *S. typhimurium* vaccine, administered 2 days after the virus as described in Materials and Methods, antibodies appeared which could be measured by the HE titration beginning 5 days after immunization (Chart 3 and Table 3). These antibodies apparently belonged almost exclusively to the IgM class until 19–29 days after immunization.

### Table 2

<table>
<thead>
<tr>
<th>Day after 2° immunization</th>
<th>Infected</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG + IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>2</td>
<td>3.658 ± 1.136</td>
<td>0.432 ± 0.632</td>
</tr>
<tr>
<td>4</td>
<td>6.858 ± 2.375</td>
<td>0.432 ± 0.632</td>
</tr>
<tr>
<td>6</td>
<td>8.489 ± 3.071</td>
<td>0.303 ± 0.753</td>
</tr>
<tr>
<td>9</td>
<td>7.189 ± 2.061</td>
<td>0.532 ± 0.343</td>
</tr>
<tr>
<td>12</td>
<td>7.122 ± 1.135</td>
<td>0.864 ± 0.843</td>
</tr>
<tr>
<td>17</td>
<td>7.022 ± 2.452</td>
<td>1.397 ± 0.577</td>
</tr>
<tr>
<td>21</td>
<td>5.225 ± 2.035</td>
<td>1.397 ± 0.577</td>
</tr>
<tr>
<td>34</td>
<td>4.225 ± 1.604</td>
<td>0.432 ± 0.632</td>
</tr>
</tbody>
</table>

Secondary antibody response in latent leukemia, mean log₂ titer reciprocals ± standard deviation. Data shown in Chart 2.
since they could not be measured in ME-treated sera by the HA test. A single animal of the ten uninfected control mice showed antibody by the latter criterion at 19 days after immunization. Three of the control and 2 of the infected animals had IgM antibody titers at termination of the experiment 29 days after immunization. Only the curves for mean titers of HE antibodies are given in Chart 3 because it was felt that the titers on 2-ME treated sera were too low to provide a basis for definite conclusions, except to say that infected mice can produce antibodies of the IgG type.

The extent of immunosuppression due to the virus is evident. By Day 19 after immunization, when the mean titer of the uninfected group was slightly less than 5.0 log₂ units, with 8 of 10 animals responding, that of the infected animals was less than 1.0 units, based on a single animal responding at the level of 2.322 log₂ units. The peak titer reached by the uninfected controls within the time limit of the experiment was about 16-fold (arithmetically) higher than that of the infected group. The peak titer of the infected group was reached 12 days after immunization and fell sharply to almost zero during the ensuing week. The subsequent rise in mean log₂ titers of the infected group appeared to be due to a late response on the part of individual mice due partly, but not entirely, to IgG.

Two indices were used to compare development of immunologic impairment and splenomegaly: the immunosuppressive index (see above) and the splenomegaly index, obtained by dividing the mean spleen weight of the normal uninfected mice by that of the infected animals. The upper part of Chart 4 shows that, in general, the indices paralleled each other. However, during Phase II, the slope of the immunosuppressive

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**Table 3**

<table>
<thead>
<tr>
<th>Day after Immunization</th>
<th>Infected</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgM + IgG</td>
<td>IgG</td>
</tr>
<tr>
<td>8</td>
<td>0.532 ± 0.948</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>1.197 ± 1.258</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>0.232 ± 0.835</td>
<td>0</td>
</tr>
<tr>
<td>29</td>
<td>1.629 ± 1.093</td>
<td>0.964 ± 1.080</td>
</tr>
</tbody>
</table>

Primary antibody response in latent leukemia when virus is administered two days before immunization. Mean log₂ titers ± standard deviation. Data shown in Chart 3.
Effect of "Attenuated" Friend Virus on the Primary Response When Virus Is Administered after Immunization. In this experiment, the virus was administered 2 days after the antigen. As shown in Chart 5 and Table 4 and the lower part of Chart 4, on the first day of bleeding, 5 days after immunization, and 3 days after infection, the HE titers of the infected mice average about 0.7 log₂ units higher than those of the controls, with a correspondingly elevated immunosuppressive index (>1.0). It is not known whether this apparent stimulating effect of the virus is significant. Otherwise, the mean titers of the infected animals failed to reach the levels of the controls at later time intervals. The impairment in antibody response was, however, not as severe as that observed when the virus was given before the vaccine. As shown in the lower part of Chart 4, the immunosuppressive index paralleled the splenomegaly index.

Effect of "Attenuated" Friend Virus on the Primary Response When Virus Was Administered Seven Days after Immunization. In this experiment, immunization with a single dose of S. typhimurium vaccine preceded infection with the virus by 7 days, so that measurable antibody response was already present from the onset of infection (Table 5, Chart 6). Beginning at Day 12, 5 days after injection of the virus, a moderate degree of suppression of the total antibody response was noted. The IgG response was significantly suppressed throughout its detectable range beginning at Day 19, 12 days after injection of the virus.
### Table 4

<table>
<thead>
<tr>
<th>Day after immunization</th>
<th>Infected</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM + IgG</td>
<td>IgG</td>
</tr>
<tr>
<td>5</td>
<td>2.293 ± 0.463</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>2.825 ± 0.951</td>
<td>0</td>
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<tr>
<td>12</td>
<td>2.029 ± 0.387</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>0.530 ± 0.949</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>1.500 ± 1.438</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0.432 ± 0.664</td>
<td>Trace</td>
</tr>
</tbody>
</table>

Primary antibody response in latent leukemia when virus is administered two days after immunization. Mean log₂ titers ± standard deviation. Data shown in Chart 5.

### Table 5

<table>
<thead>
<tr>
<th>Day after immunization</th>
<th>All animals</th>
<th>Infected</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM + IgG</td>
<td>IgM + IgG</td>
<td>IgM + IgG</td>
</tr>
<tr>
<td>4</td>
<td>1.744 ± 1.405</td>
<td>2.361 ± 0.894</td>
<td>2.761 ± 0.447</td>
</tr>
<tr>
<td>6</td>
<td>2.943 ± 0.793</td>
<td>4.790 ± 1.118</td>
<td>4.658 ± 1.773</td>
</tr>
<tr>
<td>7</td>
<td>Day of infection</td>
<td>4.580 ± 0.886</td>
<td>6.190 ± 1.014</td>
</tr>
<tr>
<td>12</td>
<td>3.760 ± 1.060</td>
<td>1.730 ± 1.032</td>
<td>4.860 ± 0.886</td>
</tr>
<tr>
<td>19</td>
<td>2.816 ± 1.176</td>
<td>0.960 ± 1.225</td>
<td>5.823 ± 1.262</td>
</tr>
</tbody>
</table>

Primary antibody response in latent leukemia when virus is administered seven days after immunization. Mean log₂ titers ± standard deviation. Data shown in Chart 6.

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Chart 6. Primary antibody response in latent leukemia when virus is administered seven days after immunization. Each point represents the mean log₂ titer of 10 animals. Number of responders per 10 mice is shown at each point.
DISCUSSION

The immunosuppressive effects of murine leukemia viruses are now well documented. The primary circulating antibody response, as measured by direct hemagglutination or hemolysis, was depressed when antigen (sheep erythrocytes, SRBC) was given on the same day as the virus or later, with slight depression of peak titer when antigen was given 2 days before the virus (4). This result was noted with high doses of Friend virus (100–500 ID50) when both virus and antigen were given intraperitoneally. A similar experiment with Rauscher virus, but with an unspecified dose (undiluted 10% acellular splenic extract), produced no significant depression of hemagglutinin response when antigen was given during the interval 4 days before to 3 days after the virus, both antigen and virus being given i.p. (12). Slight inhibition of the response to SRBC given intravenously was noted when Friend virus (undiluted 10% spleen homogenate) was given i.p. 1 day after the antigen; this suppression becoming more marked with increasing intervals between administration of antigen and virus (19).

Salaman and Wedderburn (27) reported results which in general coincided with the above, but they were somewhat difficult to interpret because of variations in methods of preparation of virus suspensions and selection of route of antigen injection. However, these authors came closer than others to using a low dose of Friend virus (1:512 dilution of infected mouse plasma) which they stated produced palpable splenomegaly only after 28 days. This virus dose had no immunosuppressive effect when the antigen was given 4 days after the virus. However, immunosuppression by higher doses of the same virus preparation were transient.

For the most part, investigations have been conducted with doses of Friend and Rauscher virus which may be assumed to be high, because of the use of undiluted spleen homogenates and also because of spleen enlargement within a short period of a few days to a week or so. When given by the i.p. route, these viruses appear to exert their immunodepressive effects on the primary response to SRBC only when given before or very nearly concurrent with, the antigen. On the other hand, mice injected i.p. with beef serum albumin (BSA) in complete Freund's adjuvant, and infected 3 or 10 days later with Rauscher virus, had a depressed response to BSA (31). It is doubtful whether this can be considered a true primary response.

During latent stages of Moloney virus disease, even during the total absence of signs or symptoms of leukemia, there was a depressed ability to respond to SRBC (7). This was true also with respect to the ability of C3H mice to respond to phage T2 during the latent period of Passage A leukemia (21). These infections perhaps exert their effect through the thymus gland (5).

In our experiments reported above, interference was observed with an ongoing primary response to salmonella vaccine even when the antigen was administered 7 days before the virus, involving also the later appearing IgG antibody response. In all of the reports examined above, the virus was presented to the host by the i.p. route, whereas we have used the i.v. route for virus and i.p. injection for antigen. It is known that the disease proceeds more rapidly following i.v. injection (26).

Thus, the differences in reactivity involving depression of an ongoing established primary response in these experiments, and the failure (with the exception of the results reported by Morton and Siegel) to demonstrate such interference as discussed above, may be due to (a) differences in antigen used (salmonella "O" vaccine vs SRBC); (b) route of administration of virus (i.v. vs i.p.); (c) possibly host strain (random-bred vs inbred mice); (d) characteristics of the virus (attenuated or low dose vs virulent high dose). Further research may clarify this problem.

Our data show that immunodepression is more apparent in the IgG response for the secondary response than for the IgM + IgG response; otherwise, the results are similar to those obtained by others, e.g., the secondary response was less affected than the primary response.

Space does not permit inclusion of discussion of results obtained by various laboratories with the localized hemolysis-in-gel technic (2–4, 10, 33). In general, these results have tended to corroborate those obtained using measurement of circulating antibodies. We intend to pursue studies of this sort in the future, however, because spleens of mice infected with FV6H virus are not extensively necrotic nor infiltrated with neoplastic cells prior to 3 weeks, which is usually the case with more active preparations.

The division of the disease produced by FV6H into three phases was conveniently arrived at by considering the various objective parameters available. However, the splenomegaly indices displayed in Chart 7 indicate a definite downward trend through Phases I and II beginning about 7 days after the virus was administered. It must be conceded that the activities of the virus begin soon after injection, and that the attenuation is a relative matter with virus FV6H. More prolonged latency periods with both the Friend and Rauscher virus are at present being sought in this laboratory and will be discussed in future publications.

REFERENCES


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