Role of Hemopoietic Colony-Forming Cell Responses in the Pathogenesis of Ectromelia

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Intraperitoneal injection of complete Freund’s adjuvant which caused an extensive cellular response in the reticuloendothelial system greatly increased the severity of infection which resulted from footpad inoculation of ectromelia virus in mice. The mortality was greatest during the proliferative phase of the cellular response, indicated by the colony-forming cell response. Exposure of colony-forming cells to virus in vitro showed that those from normal bone marrow were much less susceptible to virus than those from the bone marrow of adjuvant-treated mice and more particularly those from the spleen of adjuvant-treated mice. Severe bleeding to induce anemia and a consequent erythropoietic response did not increase the mortality from ectromelia. Previous results were interpreted on the basis of these findings and a more general application of them to the pathogenesis of poxvirus infection was discussed.

It was reported in the previous paper (13) that during infection with ectromelia virus mice can develop an increase in the number of hemopoietic colony-forming cells (CFC) in bone marrow, blood, and spleen. However, it was also shown that the relationship between the level of infection and the CFC response in the different tissues was complex, and that this relationship in bone marrow was different from that in spleen and blood (13). No clear-cut pattern emerged regarding the possible role of a CFC response in the pathogenesis of the disease since the results could be interpreted in various ways. The experiments reported in this paper were carried out in an attempt to resolve these problems by (i) studying the infection in mice whose CFC system was stimulated before infection and (ii) by studying the susceptibility of CFC to virus infection in vitro.

MATERIALS AND METHODS

The mice, the strain of virus, and the technique of virus and infectious cell assay were described or referred to in a previous paper (13).

Hemopoietic stimuli. Intraperitoneal injection of complete Freund’s adjuvant has been shown to give an intensive stimulus to the CFC system (12). The complete adjuvant (Difco) was used as supplied, without emulsification, but ensuring complete suspension of the mycobacterial component. Each mouse was given 0.2 ml by intraperitoneal inoculation.

A strong erythropoietic stimulus was given by making mice anemic. This was achieved by bleeding 0.3 ml from the tail on 3 successive days, a procedure which resulted in the peripheral blood hematocrit falling from 45% to 20 to 25%. Virus was given the day after the third bleeding.

Infection of CFC in vitro. Bone marrow and spleen cells were collected from either normal mice or from mice which had been given adjuvant 7 days previously. Cell suspensions were made as described in the previous paper (13) and were diluted to a concentration of \(4 \times 10^5\) per ml. A 1-ml amount of this suspension was mixed with an equal volume of Eagle’s medium containing \(4 \times 10^7\) plaque-forming-units (PFU) of ectromelia virus. The mixture was incubated at 37 C in a water bath for 2 hr with gentle shaking every 10 to 15 min. The cells were then washed, counted, and cultured for colony formation as previously described. Control suspensions from the same tissues were incubated with either medium only or with the same concentration of heat-inactivated virus. Inactivation of virus was carried out at 60 C for 1 hr.

Effect of adjuvant on mortality and the course of infection. Adjuvant was given intraperitoneally and virus by subcutaneous inoculation of the right hind footpad. The footpad route of inoculation required approximately 10 PFU of virus to kill 50% of normal mice compared with 10 PFU of the same virus given intraperitoneally.

Figure 1 shows the mortality index (per cent mortality divided by the average time in days between virus inoculation and death). Index was determined after 10 PFU of virus were given by footpad inoculation in groups of 10 mice, treated with adjuvant from 6 days before virus to 4 days after. This clearly shows
that adjuvant treatment at any time from 6 days before to 1 to 2 days after virus resulted in 10 to 30% of mice dying from a dose of virus which normally would not cause any deaths.

Figure 2 shows the proportion of infected cells in the spleens of mice given $10^9$ PFU of virus by footpad inoculation. These are mean values from groups of five mice given either no adjuvant, adjuvant 1 day, or adjuvant 7 days before virus. These data show that although adjuvant treatment did not accelerate the appearance of virus-infected cells in spleen it greatly increased their number.

**Mortality in relationship to cellular changes in spleen.**

Since ectromelia is primarily an infection of the lymphoreticular system and it is known that adjuvant causes a marked cellular proliferation in this system (18), it was of interest to determine whether the increased susceptibility to ectromelia was associated with the maximum proliferation of precursor cells (CFC) or with the later increase in numbers of mature cells. Figure 3 shows the relative timing of these cellular events in spleen and indicated that the CFC phase reached its peak about 1 week before the peak of mature granulocyte-macrophage cells and about 2 weeks before the peak of total white cells (mainly lymphocytes).

It was shown (Fig. 2) that the infection makes its appearance in the spleen 3 days after footpad inoculation of virus in adjuvant-treated animals. Therefore, if a correlation is to be made between susceptibility to infection and changing cell populations in the spleen, this 3-day interval must be taken into account; i.e., if animals are given adjuvant 6 days before virus, there is in fact a 9-day interval between adjuvant and the first appearance of virus infection in spleen. Table 1 shows the mortality in groups of 25 mice given $5 \times 10^9$ PFU of virus by footpad inoculation either 2, 6, or 10 days after adjuvant. Two control groups of 25 mice were given either virus alone or adjuvant alone, and there were no deaths in either group. This shows that adjuvant treatment was more effective if given 2 or 6 days before virus than if given 10 days before.

Allowing for the 3-day interval mentioned above, the pretreatment intervals as far as spleen infection is concerned become 5, 9, and 13 days. Comparison of these results to those shown in Fig. 3 shows that the increased mortality was associated more with the CFC response than with the increase in numbers of mature cells.

**Infection of CFC in vitro.** Three preliminary experiments on infection of normal bone marrow cells in vitro with approximately 5 PFU of virus per cell gave reductions in colony number of 5, 7, and 8%, none of
**TABLE 1. Percentage mortality in groups of 25 mice given ectromelia virus**

<table>
<thead>
<tr>
<th>Group</th>
<th>Per cent mortality</th>
<th>Time (day) between virus inoculation and death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (virus only)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Control (adjuvant only)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Adjuvant + virus (10-day interval)</td>
<td>24</td>
<td>11.0 ± 2.3</td>
</tr>
<tr>
<td>Adjuvant + virus (6-day interval)</td>
<td>68</td>
<td>11.1 ± 2.6</td>
</tr>
<tr>
<td>Adjuvant + virus (2-day interval)</td>
<td>60</td>
<td>11.0 ± 2.2</td>
</tr>
</tbody>
</table>

* Mice were given $5 \times 10^3$ plaque-forming units of ectromelia virus by footpad inoculation either 2, 6, or 10 days after a 0.2-ml intraperitoneal injection of complete Freund's adjuvant.

**TABLE 2. Change in colony-forming potential of cells from normal marrow, adjuvant-stimulated marrow and adjuvant-stimulated spleen when preincubated with ectromelia virus at a multiplicity of 8**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Colony reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal marrow</td>
<td>9</td>
</tr>
<tr>
<td>Adjuvant marrow</td>
<td>44</td>
</tr>
<tr>
<td>Adjuvant spleen</td>
<td>78</td>
</tr>
</tbody>
</table>

These being significantly different from control cell suspensions.

Preliminary experiments also showed that when spleen cells were taken from animals 6 days after adjuvant and infected in vitro there was an 80% reduc-

**Fig. 3. Cellular changes in spleen after intraperitoneal inoculation of 0.2 ml of complete Freund's adjuvant. Symbols: , total white cells; , granulocytes plus macrophages; , proportion of colony-forming cells compared with uninoculated control mice.**

**TABLE 3. Change in colony-forming potential of cells from normal marrow and adjuvant-stimulated spleen when preincubated with either live or killed virus**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Colony reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal marrow</td>
</tr>
<tr>
<td>Live</td>
<td>18*</td>
</tr>
<tr>
<td>Killed</td>
<td>6</td>
</tr>
</tbody>
</table>

* Values are the average of two experiments.

...tion in the colony-forming potential. These results were investigated in more detail by measuring the percentage reduction in colonies from normal marrow, marrow from adjuvant-treated mice, and spleen from adjuvant-treated mice when infected by the same virus preparation in vitro. The number of CFC present in normal spleens was too low to permit accurate measurement of percentage reduction. The results of such an experiment are shown in Table 2. Table 3 gives the average results of two experiments in which normal marrow cells and spleen cells from adjuvant-treated mice were preincubated with either live virus, heat-inactivated virus, or medium alone. Taken together these results confirm that normal marrow CFC were mostly insusceptible to virus in vitro and that CFC from marrow of adjuvant-treated mice were more susceptible but not as susceptible as the CFC from spleens of adjuvant-treated animals. It is also clear that part of the effect on adjuvant-stimulated spleen CFC did not depend upon the viability of the virus.

**Mortality in anemic mice.** An intraperitoneal injection of complete Freund's adjuvant gives such an intensive myelopoietic stimulus that erythropoiesis can
It seems likely that the discrepancy between spleen and bone marrow in terms of the relationship between infected cells and CFC response, which was described in the previous paper, was due to the much greater susceptibility of CFC to infection once they leave the marrow.

The precise nature of the CFC has not been established with certainty. The available evidence indicates that pluripotent hemopoietic stem cells [which can be detected by an in vivo assay (19)] give rise to classes of progenitor cells limited to particular pathways of differentiation, for example, erythropoietin-sensitive and antigen-sensitive cells (11). The in vitro CFC probably represent another class of progenitor cell restricted to granulocytic and macrophage differentiation (9).

Since CFC are present as a very small proportion of the total cells in hemopoietic tissues, it seems likely that the property of colony formation is restricted to a narrow phase of the differentiation pathway from stem cell to mature granulocyte or macrophage. In actual cell numbers, the 20-fold increase in spleen CFC at the height of the response to adjuvant represents approximately 10^6 CFC per spleen. Even at this level, the CFC comprise less than 0.05% of all spleen white cells; it seems unlikely that such a small proportion of cells, even if highly susceptible to virus, could account for the striking effects of adjuvant treatment on the levels of infection. It would be more plausible to postulate that the increase in susceptibility to virus is a property of proliferating cells over a wider range of differentiation.

The results obtained by infecting anemic mice showed that an erythropoietic response is not associated with greater susceptibility to ectromelia; therefore, this is not a feature of all proliferative cellular responses. However, there is evidence from immunofluorescence studies (15) that proliferating cells of the lymphoid series become susceptible, since in addition to phagocytic cells there is a striking localization of ectromelia infection in the follicles of lymph lobes and spleen, another site of active cellular proliferation.

Experiments with lymphocyte transformation in vitro have shown that the transformed cells are much more susceptible to several viruses than the small lymphocytes from which they were derived (3, 4, 17). Recent reports on the development and histopathology of Rauscher leukemia virus infection again suggest that the early infection is associated with proliferative responses in lymphoid tissues (7, 8). Interpretation of such findings with oncogenic viruses is difficult since it is not clear whether there is a normal proliferative response to infection resulting in increased susceptibility of proliferating cells which then become

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**TABLE 4. Per cent mortality in a group of 20 anemic mice compared with 20 normal mice given ectromelia virus**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Mortality (%)</th>
<th>Mean time of death (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>40</td>
<td>9.9 ± 1.1</td>
</tr>
<tr>
<td>Anemic</td>
<td>30</td>
<td>9.2 ± 1.3</td>
</tr>
</tbody>
</table>

* Mice were given 2 x 10^4 plaque-forming units intraperitoneally.
transformed into tumor cells, or whether the lymphoid proliferation is abnormal from the beginning. However, the observation that primunization of the animals with sheep erythrocytes can potentiate the effect of Rauscher virus supports the former interpretation (8).

In addition to its pronounced myeloproliferative effect, Freund's complete adjuvant has other known and probably some unknown effects. It is of course a potent immunological adjuvant and this effect is demonstrable even if the antigen is given at a different time or by a different route (2, 10, 16). As far as ectromelia infection is concerned, an adjuvant effect would be expected to help the animal overcome infection rather than the reverse. Another relevant effect reported recently is that pretreatment with adjuvant can alter subsequent interferon responses (1). This is unlikely to have played a significant role in the present experiments since it was shown that adjuvant increases mortality from ectromelia when given at the same time or even 1 to 2 days after inoculation of virus (Fig. 1), and this would coincide with the phase of enhanced interferon responsiveness. Another possibility is that the increased mortality in ectromelia was due to some toxic effect of the adjuvant. This is a proposition which by its very imprecision is impossible to rule out. It has, however, been shown that in other virus infections adjuvant may have either no effect (1) or may inhibit virus multiplication (6). Increased mortality in ectromelia also occurs in mice treated with bacterial endotoxin (5). Like complete Freund's adjuvant, endotoxin has many biological effects, but it is interesting that one of these is a marked CFC response (12).

If the hypothesis presented here regarding the increased virus susceptibility of proliferating cells in the lymphoreticular system is correct, a further anomaly requires explanation. In the previous paper it was shown that mice with the highest CFC responses in spleen and blood had relatively low levels of infection (13). It was suggested that these animals developed the infection to a sufficient degree to stimulate a CFC response and had subsequently overcome the infection. However, in the light of the adjuvant experiments, a brisk CFC response should have made the mice less likely to overcome infection.

This anomaly serves to emphasize an important concept, namely that the outcome of an infection may depend upon a fine balance between host responses which have opposite effects, particularly when such responses are interrelated. Other experiments to be reported from this laboratory have shown a very close correlation between immune responsiveness and CFC responsiveness when compared by age, sex, and strain of mouse. The infected mice referred to above with a high CFC response and mild infection were probably protected by a brisk immune response which was accompanied by a brisk CFC response.

The delicate balance of cellular responses influencing the outcome of ectromelia infection is further emphasized by the likelihood that not only are immune responsiveness and CFC responsiveness related physiologically, one leading to protection and the other to susceptibility, but the immune responses which are eventually protective also depend upon proliferative cellular responses which may give a period of greater susceptibility during their development.

Given a basic situation such as this, it would be expected that many factors which by themselves may be relatively minor could assume major importance when the experimental conditions allow them to upset this balance in one direction or the other. Among such factors may be small variations in the dose of virus, the strain of virus (differences in growth rate), the strain of host, the route of inoculation or the action of an antiviral drug.

ACKNOWLEDGMENT

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LITERATURE CITED