In vitro Immune Responses of Spleen Cells from Friend Virus Infected Mice

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SUMMARY

The primary immune response to sheep erythrocytes of spleen cells cultured from mice infected with Friend virus was compared with that of normal spleen cell cultures. Significant depression of the response, as measured by the production of haemolytic plaque-forming cells, was first observed when mice had been infected for 3 days prior to cell culture. This depression became more severe as the infection period was prolonged.

After 4 days infection in vivo the peak of the in vitro response was reduced to a mean of 11% of normal values. No depression was observed during the first 48 hr of the development of the response in such cultures.

The immune defect was shown to exist only in the non-adherent (lymphocyte-like) fraction of the spleen.

INTRODUCTION

Mice infected with Friend virus (Friend, 1957) produce depressed primary and secondary responses in vivo to sheep erythrocytes (Old et al. 1960; Odaka et al. 1966; Salaman & Wedderburn, 1966; Wedderburn & Salaman, 1968; Cegłowski & Friedman, 1968; Bennett & Steeves, 1970). The response to a number of other antigens is also depressed (Millian & Schaeffer, 1968; Hirano et al. 1969). It would appear that the virus acts principally on the more primitive elements of the haematopoietic system (Mirand, 1967; Friedman & Cegłowski, 1968; Chan et al. 1968) but the mechanism of the immune depression is not fully understood.

We have studied the effect of Friend virus infection in vivo on the primary immune response to sheep erythrocytes in vitro, using the spleen culture technique of Mishell & Dutton (1967). In this system a minimum of three cell types are believed to co-operate in the production of the response (Mosier & Copplestone, 1968; Haskell, Byrt & Marbrook, 1970; Dutton et al. 1970; Tan & Gordon, 1971). To study the effect of Friend virus on these co-operating cells we have used the cell-adherence separation technique of Mosier (1967).

METHODS

Animals. Two to 6-month-old Balb/c mice of either sex were used for all experiments. Mice in any one experiment were of the same sex and differed in age by not more than 1 week.

Antigen. Sheep erythrocytes (SE) from selected individual donors were obtained from Wellcome Reagents and stored in the cold for 2 weeks before use.
Friend virus. Complete details of the preparation of Friend virus (FV) were given in a previous paper (Wedderburn & Salaman, 1968). Mice were infected intravenously with 0.1 ml. doses of citrated plasma or spleen extract containing $10^4$ ID$_{50}$ of the virus.

Media. Foetal calf sera were obtained from Flow Laboratories and from Armour Pharmaceuticals. Batches were selected for their ability to support the in vitro immune response of spleen cells and stored at $-20^\circ$. Eagle’s MEM was obtained from Wellcome Reagents; supplements from Flow Laboratories and from BDH Chemicals.

Culture medium, nutritional mixture, and balanced salt solution (BSS) were prepared as described by Mishell & Dutton (1967), except that 40 µg./ml. L-asparagine was added to the culture medium. Eagle–Hepes (Eagle’s MEM buffered with 10 mM-HEPES (BDH Chemicals)) was used to prepare the cells for culture, and to stabilize the pH of the culture medium during cell separation and harvesting.

Cell culture. Mice were killed by cervical dislocation. Their spleens were cut into three or four pieces and pressed gently through a fine mesh stainless-steel sieve using Eagle–Hepes containing 30% inactivated calf serum (Wellcome Reagents) as diluent. Clumps of cells were dispersed by repeated aspiration through a Pasteur pipette; any aggregates remaining were allowed to sediment. The overlying cell suspension was washed once in serum-free Eagle–Hepes and resuspended in 10 ml. complete culture medium per spleen. This suspension contained 12 to 20 x $10^6$ nucleated cells/ml with approximately 90% viability. The suspension was fractionated as described under ‘Cell separation’, or incubated directly; in the latter case 1 ml. volumes were placed in 30 mm. plastic tissue culture plates (Sterilin) with 0.05 ml. washed 10% SE in BSS. The plates were incubated at 35° on a platform rocking at 6 cyc./min. in a water-saturated atmosphere of 10% CO$_2$, 7% O$_2$, and 83% N$_2$. Cultures were fed daily with 0.1 ml. nutritional mixture.

Cell separation. Adherent macrophage-like cells (fraction M), and non-adherent lymphocyte-like cells (fraction L) were prepared by the technique of Mosier (1967), modified as follows:

One ml. samples of cell suspension were incubated in 30 mm. culture plates without rocking or the addition of SE. After 30 min. the plates were gently agitated and placed on the rocker for a further 30 min. incubation. The medium in each plate was then replaced with 2 ml. Eagle–Hepes and loose cells displaced by pipetting the liquid over the surface of the plate. The resulting cell suspension was discarded and the washing procedure twice repeated, leaving an even layer of firmly attached cells (fraction M). Microscope counts of 10 random fields/plate showed, over numerous experiments, a range from 3 to 9 x $10^5$ adhering cells/plate. In any one experiment all plates contained approximately the same number of adherent cells.

Fraction L was prepared by incubating 10 ml. volumes of suspension (adjusted to contain 15 x $10^6$ viable nucleated cells/ml.) in 90 mm. plastic tissue culture plates (Sterilin) on the rocker for 30 min. without the addition of SE. Some 7 to 8 ml. of non-adherent cell suspension was then carefully removed from each plate and transferred to fresh 90 mm. plates, which were rocked for a further 30 min. Each plate finally yielded 6 to 7 ml. of suspension containing some 50 x $10^6$ viable non-adherent cells.

These were centrifuged at 250 g for 5 min. and resuspended in fresh culture medium to the original cell concentration. Samples of 1 ml. were transferred either to fresh 30 mm. plates or to plates containing fraction M cells, which, after the addition of SE, were incubated as previously described.

Haemolytic plaque assay. Cultured cells were harvested into cold Eagle–Hepes (using a policeman to remove adherent cells), centrifuged in the cold at 250 g for 5 min. and resus-
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Pended in an appropriate volume of cold BSS. Haemolytic plaque-forming cells (PFC) were enumerated by the technique of Jerne & Nordin (1963), modified by the use of BSS in both agar layers.

Cell counts. Viable nucleated cells were enumerated directly in a haemocytometer, taking the exclusion of 0.05 % nigrosin in Ringer's solution as evidence of viability. Cell counts were performed on each suspension prepared for culture, and on each culture prepared for PFC assay.

Experimental design. Groups of two to ten mice were used, depending on the number of plates required. Two or four plates were prepared for each variable; these were harvested in pairs and assayed in triplicate for PFC. The mean number of plaques in each set of assay plates was expressed as PFC/10^6 viable nucleated cells, as counted in the suspension prepared for assay.

Where differences between experimental data are claimed, they have been established as statistically valid by means of the 'Student' t test for small samples with a selected value for p of 0.05.

RESULTS

Primary response of infected cells

Preliminary experiments were performed to determine whether the primary response to SE in vitro was depressed as a result of FV infection in vivo. Groups of mice were infected at intervals ranging from 1 hr to 7 days before their spleen cells were cultured. These cultures, together with control cultures from uninfected mice, were assayed for PFC against SE after 5 days incubation with SE ('stimulated'), and without SE ('unstimulated'). Unstimulated cultures were included since PFC against SE develop in excess of background in the absence of this antigen. This may be due to cross-reacting antigens in the foetal calf serum included in the culture medium (Mishell & Dutton, 1967; Marbrook, 1967).

Fig. 1 shows that after a minimum of 3 days infection in vivo the response in vitro was depressed, and that this depression increased as the in vivo infection period was extended. These findings are comparable to, though not identical with, those made in vivo (Wedderburn & Salaman, 1968). The effect of FV on unstimulated PFC levels is also shown in Fig. 1; although variations both above and below the corresponding control range were observed, these were at most only marginally significant.

Primary responses of spleen cells from 4-day infected mice

There is evidence that cell transformation occurs as early as 1 day after infection in vivo with FV (Rossi, Cudkowicz & Friend, 1970), and definite morphological and histological changes in the spleen are detectable 4 days after infection (Metcalf, Furth & Buffet, 1959; Dawson, Fieldsteel & Bostick, 1963). The spleen enlarges throughout the course of the disease as the number of nucleated cells it contains increases. This increase is due mainly to the abnormal proliferation of erythroid precursors. Thus it becomes progressively less meaningful to relate the response of an infected suspension to that of a normal suspension containing the same number of cells, since the ratio of immunocompetent cells to total cells in the former may have been reduced by this proliferation of non-lymphoid cells. We have observed that sub-maximal PFC levels were obtained even when normal cells were cultured at concentrations in excess of 2 x 10^7/ml. As it was not possible to culture equivalent fractions of normal and infected spleens without exceeding this concentration in cultures from mice infected for more than 4 days, we have worked routinely with spleens from 4-day infected mice. The weight and total cell number of such spleens did not exceed those of normal spleens.
by more than 10%, so that comparisons between PFC counts per million viable cells may still be meaningful. Hereafter the term ‘infected cells’ is used to denote spleen cells from mice infected with FV 4 days prior to culture.

Table 1 gives the results of ten representative experiments in which the 5-day primary responses of normal and infected cultures were compared. Although there was considerable variation in PFC numbers between experiments, the stimulated response of infected cultures was always markedly depressed in relation to the comparable uninfected control. Once again FV only slightly affected the unstimulated response.

The degree of immune depression exerted by FV was similar whether the results were expressed as PFC/10⁶ cells originally cultured, or as PFC/10⁶ cells surviving on day 5, since

![Graph showing effect of FV infection on immune response]

**Fig. 1.** Effect of length of infection *in vivo* on subsequent primary response to SE *in vitro*. *, Viable nucleated spleen cells after 5 days incubation. ––, FV-infected SE-stimulated. - - - - - - , FV-infected, no SE added to cultures. Parallel lines indicate range of responses of uninfected cultures.

<table>
<thead>
<tr>
<th>Expt</th>
<th>SE-stimulated</th>
<th>No SE to cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Infected</td>
</tr>
<tr>
<td>19</td>
<td>780</td>
<td>44</td>
</tr>
<tr>
<td>22</td>
<td>492</td>
<td>20</td>
</tr>
<tr>
<td>32</td>
<td>325</td>
<td>12</td>
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<tr>
<td>36</td>
<td>497</td>
<td>127</td>
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<tr>
<td>37a</td>
<td>429</td>
<td>131</td>
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<tr>
<td>37b</td>
<td>840</td>
<td>42</td>
</tr>
<tr>
<td>45</td>
<td>726</td>
<td>86</td>
</tr>
<tr>
<td>47</td>
<td>247</td>
<td>55</td>
</tr>
<tr>
<td>51</td>
<td>848</td>
<td>179</td>
</tr>
<tr>
<td>52</td>
<td>574</td>
<td>84</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>536</td>
<td>59</td>
</tr>
<tr>
<td>% of normal</td>
<td>100</td>
<td>11</td>
</tr>
</tbody>
</table>

**Table 1.** Primary response to SE *in vitro* of spleen cells from normal and 4-day FV-infected mice

PFC/10⁶ viable cells assayed after 5 days culture
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there was similar cell survival (approximately 25%) in normal and infected cultures. However, as viable cell counts did not distinguish between different nucleated cell types, it could not be determined whether the ratio of immunocompetent cells to total surviving cells was the same for both categories. If the results were expressed as PFC/spleen, as in previous in vivo studies (Wedderburn & Salaman, 1968), the degree of immune depression was not materially altered. For example, in Expt 35 (Table 1) the normal and infected responses were respectively 726 and 86 PFC/10^8 cells assayed, and respectively 25 400 and 2800 PFC/spleen; a ratio of approximately 8:1 in both cases.

Fig. 2. Development of primary responses to SE in vitro of spleen cells from normal and 4-day FV-infected mice. *, Viable nucleated spleen cells assayed on day indicated. ●—●, Normal, SE-stimulated. ○—○, Normal, no SE added to cultures. ▲—▲, FV-infected, SE-stimulated. △—△, FV-infected, no SE added to cultures.

Fig. 2 shows the results of one of three similar experiments which followed the course of the response in normal and infected cultures. Culture suspensions were prepared from the pooled spleens of eight normal and eight infected mice. Four cultures for each category (normal, infected, stimulated, unstimulated) were assayed each day for a week. During the first 48 hr there were no significant differences in the rates of increase of PFC. After this time while PFC counts in stimulated normal cultures continued to rise rapidly those in all other categories rose more slowly and declined earlier.

Variation in primary responses

The experiments contributing to Table 1 were performed over more than a year, during which time may different batches of mice were used. In addition, while culture methods remained unchanged, at least two batches of virus and of foetal calf serum were used, and
the SE donor changed three times. Because such changes were from time to time inevitable, it was important to determine whether the varying experimental results seen in Table 1 were due to changes in reagents (serum, virus, antigen) or to differences in antigen responsiveness between the various groups of mice used, and to determine the extent of individual variation within such groups.

Mice for all experiments were weaned and sexed at 4 weeks of age and maintained in holding trays in batches of 30 or more. Mice from two such holding trays, arbitrarily called group A and group B, were used for the experiment shown in Table 2. Normal mice from group B, 11 weeks older than group A, had twice given significantly greater responses than the latter. However, preliminary experiments, which tested pairs of mice from every group of mice then between 2 and 6 months old (including A and B), indicated that the size of the PFC response is age- (and sex-) independent.

Table 2. Individual and group variation in primary responses to SE in vitro of spleen cells from normal and 4-day FV-infected mice

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Group A normal</th>
<th>Group B normal</th>
<th>Group A infected</th>
<th>Group B infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+SE -SE*</td>
<td>+SE -SE</td>
<td>+SE -SE</td>
<td>+SE -SE</td>
</tr>
<tr>
<td>1</td>
<td>313 75</td>
<td>977 102</td>
<td>93 47</td>
<td>70 21</td>
</tr>
<tr>
<td>2</td>
<td>552 89</td>
<td>887 49</td>
<td>151 21</td>
<td>21 9</td>
</tr>
<tr>
<td>3</td>
<td>617 116</td>
<td>815 55</td>
<td>93 47</td>
<td>59 21</td>
</tr>
<tr>
<td>4</td>
<td>318 34</td>
<td>705 44</td>
<td>222 25</td>
<td>36 7</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>429 72</td>
<td>840 59</td>
<td>131 33</td>
<td>42 13</td>
</tr>
<tr>
<td>Mean log PFC/10⁶</td>
<td>2.63 1.86</td>
<td>2.92 1.77</td>
<td>2.12 1.52</td>
<td>1.62 1.11</td>
</tr>
<tr>
<td>± Standard error</td>
<td>0.08 0.11</td>
<td>0.03 0.08</td>
<td>0.09 0.09</td>
<td>0.12 0.12</td>
</tr>
</tbody>
</table>

* No SE added to culture.

Eight mice were taken at random from each group: 4 animals from each were infected with FV 4 days before culture, the remainder acting as normal controls. Stimulated and unstimulated cultures were prepared from each spleen and assayed for PFC after 5 days incubation. Table 2 shows that, in agreement with previous experiments, the stimulated response of normal mice in group B was significantly higher than in group A. However, the stimulated response of infected mice was less depressed in the latter group. Secondly, the infected unstimulated response was significantly depressed in group B but not in group A. Thirdly, individual variation was fairly small, being greatest in cultures from infected mice.

These results show that the differences in peak PFC numbers and in the degree of depression between experiments was due at least in part to variations between the groups of mice used, and demonstrated the necessity that, as in the experiments forming Table 1, mice for any one experiment be taken from the same weaning group.

Responses of separated and recombined spleen fractions

Having established that the depression observed in vitro was essentially similar to that seen in vivo, we next attempted to locate more precisely the site of the immune defect in infected cultures. This was achieved by separating and variously recombining adherent macrophage-like cells (fraction M) and non-adherent lymphocyte-like cells (fraction L) from normal and infected spleens. The results are shown in Table 3.

No fraction cultured alone produced a significant response compared with that of the
unseparated suspension from which it was derived, although L fractions from normal primed mice produced more PFC than L fractions from normal unprimed mice. In our hands the secondary response \textit{in vitro} of mice 4 months after exposure to a low primary dose of antigen (10^6 SE intravenously) was qualitatively similar to the primary response \textit{in vitro}. Similar findings have been made by other workers (Radcliffe & Axelrad, 1971; A. Munro, personal communication).

**Table 3. Response to SE \textit{in vitro} of separated and recombined spleen cell fractions from normal and 4-day FV-infected mice**

<table>
<thead>
<tr>
<th>Cell status and treatment</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
<th>Expt 4*</th>
<th>Expt 5*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>726</td>
<td>247</td>
<td>182</td>
<td>1803</td>
<td>1064</td>
</tr>
<tr>
<td>M alone†</td>
<td>2</td>
<td>10</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>L alone</td>
<td>56</td>
<td>14</td>
<td>6</td>
<td>169</td>
<td></td>
</tr>
<tr>
<td>M + normal L</td>
<td>1066</td>
<td>843</td>
<td>203</td>
<td>899</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>86</td>
<td>55</td>
<td>76</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>M alone†</td>
<td>17</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>L alone</td>
<td>13</td>
<td>15</td>
<td>4</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>M + infected L</td>
<td>97</td>
<td>91</td>
<td>53</td>
<td>101</td>
<td>72</td>
</tr>
<tr>
<td>Normal M + infected L</td>
<td>95</td>
<td>141</td>
<td>56</td>
<td>73</td>
<td>37</td>
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<tr>
<td>Infected M + normal L</td>
<td>850</td>
<td>699</td>
<td>267</td>
<td>3134</td>
<td>1092</td>
</tr>
</tbody>
</table>

* Mice primed with one intravenous injection of 10^6 SE 4 months previously.
† PFC per culture.

Combinations of uninfected M and L fractions responded as well as or better than the suspensions from which they were derived. Combinations of infected M and L fractions, and of normal M and infected L fractions, showed the same depressed response as an unseparated infected suspension. However, infected fraction M plus normal fraction L responded as well as or better than a normal suspension, either original or recombined. This clearly indicates that the immunologically incapacitated cell type is located in the lymphocyte population (fraction L) of an FV-infected spleen. It also shows that infection with FV \textit{in vivo} does not affect that part of the \textit{in vitro} immune response which is mediated by macrophages.

**DISCUSSION**

The immune response of spleen cells from mice infected \textit{in vivo} with FV, and stimulated \textit{in vitro} with SE, was found to be depressed when virus was injected 3 or more days before the cells were taken for culture. The relationship between time of infection and degree of depression was similar to that found \textit{in vivo}, despite the fact that a number of influences which may act on spleen cells in the intact animal must be absent from the simpler \textit{in vitro} environment. That a similar depression of the immune response is not always observed in \textit{in vivo} and \textit{in vitro} systems was shown recently by Waterston (1970), who found that prior injection of pig erythrocytes depressed the \textit{in vivo} PFC response to SE, while enhancing its counterpart \textit{in vitro}. This result is further evidence against the possibility that FV depresses the immune response by acting as a competing antigen, which was discussed in a previous paper (Wedderburn & Salaman, 1968) and judged unlikely on our \textit{in vivo} results.

The dilution of immunocompetent cells during the later stages of the disease was largely
avoided by working with mice which were injected with FV 4 days before their spleen cells were taken for culture. However, it should be pointed out that, in vivo at least, if all the immune competent cells in a normal spleen are assumed to be still present, although diluted, these cells are giving a severely depressed response throughout the disease. In this respect FV differs from some other infective agents such as the Rowson Parr virus (Carter et al. 1970), murine cytomegalovirus (Osborn, Blazkovec & Walker, 1968) and a murine plasmodium (Salaman, Wedderburn & Bruce-Chwatt, 1969) for all of which the period of severe immune depression is brief.

The number of PFC per spleen produced in the primary immune response to SE in vivo was shown not to be depressed in a FV-infected mouse until more that 48 hr had elapsed after antigenic stimulation (Wedderburn & Salaman, 1968). A rather similar effect was observed in the in vitro system: no significant differences were found between the responses of stimulated or unstimulated, infected or uninfected cultures for the first 48 hr. The question whether the same mechanism underlies both these effects must await the results of further experiments, particularly those designed to elucidate the nature of the unstimulated response in vitro.

Using the differential adherence cell-separation technique first described by Mosier (1967) we have established that the immune defect in FV infection is located in the lymphocyte population (non-adherent fraction) of the spleen. Biano et al. (1971), using the same technique, have shown that the immune defect in the spleens of tumour-bearing mice is also located in this fraction.

Odaka & Köhler (1965) were able to induce persistent FV infection of macrophages in vitro, and Odaka et al. (1966) suggested that FV infection might interfere with antigen processing by macrophages. We have not determined whether macrophages from infected spleens carry virus, but our results show that, infected or not, their immunological function was not impaired. If these cells were infected, the immune competence of the normal lymphocytes with which they were combined was not altered by cross-infection.

The nature of the defect in the immunocompetent cells of spleens of FV infected mice is not yet understood. The neoplastic cell or cells appear to belong to the erythroid series. However, Hanna, Szakal & Tyndall (1970), who investigated the histological effect of the very similar Rauscher virus, reported that the earliest proliferation of the virus occurred in immunoblasts of the germinal centres. Chan et al. (1968), who looked at the ultrastructure of spleens of normal and FV-infected mice which had received SE, showed that while normal spleens contained plasma cells and immature lymphocytes, in infected spleens there were many immunoblasts containing virus particles together with a few plasma cells and lymphocytes in which no virus was seen.

There is evidence (Friedman & Ceglowski, 1968; Bennett & Steeves, 1970) that the function of a bone-marrow-derived spleen cell is affected by FV. The in vitro system described above has been shown by several authors to require the co-operation of at least three different types of cells – one adherent cell and two different non-adherent lymphoid cells, one of which is probably thymus, and the other bone marrow, derived. Experiments are now in progress which are designed to determine whether the immune functions of either or both of these types of splenic lymphocytes are affected by FV.

This work would not have been possible without the advice and encouragement of Professor J. L. Turk and Drs A. Munro, Philippa Hunter, M. R. Salaman, E. M. Tucker and H. Festenstein, to all of whom we extend our sincere thanks. We wish also to thank Mr C. Turton and Miss Diana Bullen of Wellcome Reagents Ltd for the provision of erythrocytes from
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