PRIMARY ANTIBODY RESPONSE IN ORGAN CULTURES*

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Ever since Carrel and Ingebrigsten reported limited success in the induction of immune responses in vitro (1) investigators have attempted to obtain systems in which primary immune responses could be induced and maintained in vitro in a quantitative, readily reproducible manner (2). A new light was shed on the in vitro systems by the studies of Fishman and Adler (3, 4), who demonstrated that an interaction between macrophages and lymphocytes was involved in the early stages of the response, and the role of macrophages was subsequently analyzed by several groups (5-8). Consequently, a suggestion was made that the many difficulties reported previously were due to the use of culture techniques which failed to permit adequate interactions between diverse cell types.

In this context it seemed particularly attractive to study the induction of primary immune responses in vitro by the use of experimental conditions in which cell and tissue interactions had been shown to take place (9, 10) and under which successful maintenance as well as differentiation of lymphoid tissues had already been demonstrated (11-14). The general results of these experiments, indicating that antibody response to sheep erythrocytes can be induced to a detectable level in mouse spleen explants have been reported (15) and have received independent confirmation and extension (16 = 18). The present report presents a detailed analysis of this tissue culture system.

The studies indicate that phytohemagglutinin, used in our earlier experiments (15), is not an essential component of the system, that explants initially produce mercaptoethanol-sensitive antibody, followed later by demonstrable mercaptoethanol-resistant agglutinins and that puromycin and X-irradiation inhibit the appearance of antibodies in the primary responses in vitro. A description of the histological observations on explants induced to immune responses in vitro is also presented.

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**Materials and Methods**

*Mice.*—C3H/He/Au males, 2 to 4 months old were used in all of the experiments.

*Antigens.*—Sheep blood (Gibco Grand Island Biological Corporation, New York) was used in most of the experiments. Chick blood was obtained by a cardiac puncture of adult animals. Blood was preserved in Alsever's solution for 3 to 4 wk. Erythrocytes were washed by three successive centrifugations of the blood with added saline (0.85% sodium chloride solution); red blood cell (RBC) suspensions were made in saline for injections and in medium for culturing.

*Immunization.*—Mice were injected intraperitoneally with 0.5 ml of a 5% red blood cell suspension.

*Preparation of Tissue Explants.*—Spleens were cut into cross-sectioned slices, the edges were trimmed, and the central portion cut into cuboidal fragments of 1.0 X 1.0 to 1.5 X 0.5 to 0.7 mm. These dimensions, considerably larger than those described previously (15), were used in all but the first experiment. Explants consisted of approximately 1.5 X 10⁶ cells. Thymus and lymph node fragments of similar size were used.

*Culture Method.*—The filter well organ culture method was used as previously described in detail (9, 10, 15). Explants were placed in Millipore filter wells produced by gluing 25 µ thick, 0.45 µ porosity (TH) Millipore filter paper to the underside of a Plexiglas strip, to yield a chamber of 3.0 mm diameter and 1.0 mm depth. The filter mounts were suspended over 1.0 ml medium in Falcon dish 3037.¹ Medium consisted of Eagle's basal medium supplemented with 10% agamma horse serum (Hyland Laboratories, Los Angeles) and 5% chick embryo extract (prepared from 9-day embryos), with added mycostatin (50 µg/ml) and erythromycin (50 µg/ml). Tissues were overlaid with a 1% suspension of sheep red blood cells (SRBC) prepared in medium, each culture receiving approximately 6 X 10⁵ cells which subsequently settled on the filter to surround the explant. During the 1st day of culture, some cells were found around the tissue, probably due to migration or separation from the fragment. To avoid any interference of these with the detection of agglutination, tissues were routinely transferred to new filter wells on the 3rd day of culture, and overlaid again with antigen. Antigen was added twice a week, at the time when medium was changed.

*Phytohemagglutinin.*—PHA-M (Difco Laboratories, Inc., Detroit) was used as previously described (15). For in vivo treatment, mice were injected intraperitoneally at a dose of 0.2 ml 1 day before removal of tissues. In vitro treatment was performed by adding PHA to the medium at a final concentration of 1%. Such PHA-containing medium was removed from the cultures after 3 days.

*Antibody Assays.*—Agglutination was followed daily, by gently suspending the erythrocytes in the filter well with a micropipette (15). In addition, medium was collected at various time intervals to test for the presence of agglutinins. Agglutinins were assayed in serial twofold dilutions of the medium in saline incubated with an equal volume (0.1 ml) of 1% sheep erythrocytes (SRBC) in saline for 1 hr at 37°C.

*2-Mercaptoethanol Treatment.*—Erythrocytes agglutinated around the explants were gently removed with a micropipette into small Petri dishes (30 mm diameter), and incubated for 1 hr with an equal volume of 0.1 M 2-mercaptoethanol (19). Following incubation, the agglutinate was pipetted vigorously; agglutinates disrupted into cell suspensions by this treatment were scored as 2-mercaptoethanol sensitive (2-MES), whereas stable agglutinates were designated as 2-mercaptoethanol resistant (2-MER). Standard primary and secondary antibody responses tested in this manner proved the method as adequate.

*Puromycin Treatment.*—Puromycin (Sigma, St. Louis, dihydrochloride) was added to the

¹ Previously described as No. 3020 or 3010 (15).
culture medium at the time of explantation. Cultures remained in the puromycin-containing medium for 5 days.

*X-Irradiation.*—Mice were exposed to total-body irradiation at 550 R, using a GE-Maxitron X-ray unit operated at 300 kvp, 25 ma with 0.5 mm Cu and 1.0 mm Al added filtration, at a dose rate of 80 R/min, as measured in air. Spleens were removed 24 hr following irradiation.

*Histology.*—Tissues were fixed in Zenker’s, sectioned at 5 to 7 μ and stained with hematoxylin and eosin.

**EXPERIMENTAL**

Agglutinin Formation to SRBC by Spleen Cultures.—Studies reported earlier (15) demonstrated that antibody response was initiated in spleens of mice which had been injected with PHA 1 day before explantation, where 34 of 56 cultures tested responded with agglutinin formation; yet spleen explants from untreated mice failed to manifest such a detectable reaction in vitro. Although responding fragments were obtained from each of the spleens tested, some of the explants did not manifest a detectable reaction during the entire period of culture. This could have been attributed to a complete lack of response, or to a weak response at an undetectable level. Since PHA treatment enabled a detectable response under conditions in which it otherwise failed to be manifest, the assumption was made that a more intensive stimulation with PHA might increase the incidence of responding explants.

The first series of experiments was then designed to test for the incidence of agglutinin formation by spleen fragments exposed to an additional treatment of PHA in vitro.

Three groups of cultures were studied, including: (a) spleens from PHA-treated mice; (b) spleens from PHA-treated mice as in group (a), with subsequent incubation in PHA-containing medium for 3 days; and (c) spleens from untreated mice with no exposure to PHA in vitro. Cultures were prepared and treated in the same manner as in the previous experiments (15).

The results are summarized in Table I. Whereas no antibody response was detected in untreated spleens (0/58) agglutinin reaction was demonstrable in the PHA-treated cultures (52/99 and 39/55 in the first and second group, respectively). Although the additional treatment with PHA in vitro seemed to lead to some increase in the incidence of responding cultures, there were still cultures which failed to manifest a detectable response. The question was then raised whether the PHA treatment enables a response which would otherwise fail to occur, or whether the PHA enhances a weak response to a detectable level. It therefore seemed essential to ascertain if an antibody response can take place in the absence of PHA treatment. It has been mentioned before (15) that spleen explants which were not exposed to PHA underwent a gradual decrease in size, mainly caused by a loss of cells. This was not as striking following PHA
treatment. The difference in functional behavior of the cultures, namely, antibody response, could have been attributed to that difference in their morphological appearance. As a possible mechanism it was argued that the final number of immunocompetent cells might be lower in the untreated spleens, and as a consequence, the magnitude of antibody formation too low to be detected. As an alternate possibility, the total size of the explant may have been too small, for both in the development of various embryonic systems (20) as well as in antibody-forming systems (21) a critical mass or concentration of cells has been deemed essential for optimal differentiation. In addition it was argued that the dosage of SRBC (approximately $3 \times 10^6$ cells) used previously (15) might have been too high to detect presence of antibodies under these conditions. A second series of experiments was then undertaken to test whether

TABLE I

<table>
<thead>
<tr>
<th>PHA treatment</th>
<th>No. of spleens tested</th>
<th>No. of spleens responding</th>
<th>Incidence of response‡</th>
<th>Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>16</td>
<td>15</td>
<td>52/99</td>
<td>52%</td>
</tr>
<tr>
<td>In vivo and in vitro</td>
<td>6</td>
<td>6</td>
<td>39/55</td>
<td>70%</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>0</td>
<td>0/58</td>
<td>0%</td>
</tr>
</tbody>
</table>

* Tissues explanted at approximate 0.2 to 0.3 $\times$ 0.5 $\times$ 1.0 mm dimensions.
† Number of antibody-forming cultures/total.

cultures in the absence of PHA stimulation can be induced to form detectable antibodies under modified conditions, i.e. a larger explant and a reduced dose of antigen. Two experimental groups were studied, including spleens explanted from PHA-treated as compared to untreated mice. Table II represents the results of four repeated experiments. Under these modified conditions, spleens which were not subjected to PHA treatment led to antibody-forming cultures (76/130). A somewhat higher incidence was found in cultures from PHA-treated donors (104/142). To test the consistency of these results, 40 additional spleens were tested without prior exposure to PHA. Thirty-seven of these yielded positive cultures (132/297) (Table III).

In a similar way, agglutinins were detected in 69 of 94 cultures from 15 PHA-injected mice (Table III). The incidence of antibody-forming cultures obtained from individual spleens appeared to be higher in the PHA-treated groups (Text-Fig. 1, A and B). As demonstrated in Tables I to III, in all of these experimental groups some cultures failed to manifest the reaction. The variation between individual cultures originating in the same spleen was also apparent from the intensity of the reaction; as revealed by accumulation of agglutinins in the culture...
medium. Medium was collected for agglutinin assays at intervals of 2 to 3 days for a period of 20 days. It was found that agglutinins in the medium could be detected in only some of the cultures (39 of 101 tested) in which agglutination was concomitantly observed in the filter wells. The rate of agglutinin appearance in the medium was comparable to that of spleen cultures from mice injected with antigen one day before explantation (Text-Fig. 2, A and B). When

### TABLE II

**Antibody Response to SRBC by “Large-Sized” Spleen Explants from PHA-Treated and Untreated Donors: Comparative Study**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>PHA-treated donors</th>
<th>Untreated donors</th>
<th>Incidence of responders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of spleens tested</td>
<td>No. of spleens responding</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td>23/40 57.5</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
<td>54/58 93</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>5</td>
<td>12/24 50</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2</td>
<td>15/20 75</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>14</strong></td>
<td><strong>14</strong></td>
<td><strong>104/142</strong> 73</td>
</tr>
</tbody>
</table>

* Tissues explanted at approximate 1.0 × 1.0 to 1.5 × 0.5 to 0.7 mm dimensions.  
† Number of antibody-forming cultures/total.

### TABLE III

**Agglutinin Response to SRBC by Spleen Cultures from PHA-Treated and Untreated Donors: Independent Studies**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>PHA treatment</th>
<th>No. of spleens tested</th>
<th>No. of spleens responding</th>
<th>Incidence of response*</th>
<th>Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>In vivo</td>
<td>11</td>
<td>11</td>
<td>40/55 72.5</td>
<td>%</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>4</td>
<td>4</td>
<td>29/39 74.5</td>
<td>72.5</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>11</td>
<td>11</td>
<td>24/69 34.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>6</td>
<td>5</td>
<td>31/58 53.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>4</td>
<td>4</td>
<td>12/20 60.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>10</td>
<td>8</td>
<td>15/30 30.0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>5</td>
<td>5</td>
<td>36/60 60.0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>None</td>
<td>4</td>
<td>4</td>
<td>14/40 35.0</td>
<td></td>
</tr>
</tbody>
</table>

* Number of antibody-forming cultures/total.
this was compared to agglutinin formation by spleens removed from donors 1 month after antigenic stimulation and subsequently stimulated in culture, there was a remarkable difference: the latter started sooner and persisted at a high titer long after the primary induced cultures had no detectable agglutinins in the media (Text-Fig. 2, C).

*Agglutinin Formation to Chick Erythrocytes.*—To test whether agglutinin formation appearance represents a system unique to sheep erythrocytes, or whether such a response can be elicited by other antigens as well, experiments were attempted to test the response to chick erythrocytes using the same procedure as in experiments with SRBC. Three experimental groups were studied, employing exposure to PHA either in vivo, in vitro, or without PHA treatment. Chick erythrocytes were added to one-half of the number of cultures, the others serving as unimmunized controls. Table IV represents the pooled results of such repeated experiments. Agglutinin formation was obvious in spleens which were not treated with PHA and the incidence was similar to that in the group treated with PHA in vitro. Spleens from PHA-injected mice, however, lead in this system also to a somewhat higher incidence of agglutinin-forming cultures (38/59 as compared to 10/27 and 17/49) (0.1 > P > 0.05). Cultures which were not stimulated with CHRBC did not manifest a response as tested with antigen overlayer applied on the 5th day.

Further experiments were then undertaken to test the specificity of the response. It has been reported before (15) that cultures stimulated with SRBC responded only to SRBC and not to CHRBC. Since agglutinin formation could

![Text-Fig. 1. Incidence of antibody-forming cultures of spleens originating in: A, PHA-treated donors (total 44); as compared to B, spleens from untreated donors (total 74). Five cultures were tested from each individual spleen.](image-url)
Text-Fig. 2. Agglutinin titers to SRBC in media collected from spleen cultures: A, induced in vitro to a primary response; B, spleen explanted 1 day following antigen injection to donor; and C, Spleen explanted 1 month following antigenic stimulation.
be elicited in vitro with CHRBC, the question was raised whether these were antibodies specific to the chick erythrocytes or whether this represented a factor which might agglutinate sheep erythrocytes as well. Ten fragments were prepared from each of 8 spleens removed from PHA-treated mice. Five were incubated with SRBC and the others with CHRBC. Agglutinin formation was detected in a total of 24 of 40 cultures stimulated with SRBC and 26 of 39 stimulated with CHRBC. To test whether this reaction is specific to the given antigen, each of the individual explants was then dissected into two equal-sized fragments which were placed in separate filter wells. One of each of these pairs was overlaid with SRBC and the other with CHRBC; and agglutinin formation was scored on the following days. Agglutination of SRBC was detected only around fragments previously incubated with SRBC, and similarly, agglutination of CHRBC was manifested only in cultures stimulated with this antigen (Table V). This experiment therefore suggests that agglutinin formation in vitro to CHRBC is specific as previously shown for the response to SRBC (15).

Study of Antibody Formation by Lymph Nodes and Thymus Cultures.—Previous experiments were all carried out on spleen cultures. The present experiment was undertaken to test whether other lymphoid organs also show the ability to respond to antigenic stimulation under these experimental conditions. This seemed of a particular interest concerning the thymus, since earlier experiments (cf. reference 22) raised the question whether the thymus is capable of responding to an antigenic stimulation or whether failure to detect a response by the thymus tissue results from inadequate antigenic stimulation due to a barrier preventing the entry of antigen into the thymus.

Cultures were set up from mesenteric lymph nodes, and from thymus of adult mice either injected with PHA or untreated but subsequently incubated in PHA-containing medium. The results, summarized in Table VI, demonstrate

<table>
<thead>
<tr>
<th>PHA treatment</th>
<th>No. of spleens</th>
<th>Incidence of response*</th>
<th>Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experimental</td>
<td>Controls</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>17/49</td>
<td>0/49</td>
</tr>
<tr>
<td>In vitro</td>
<td>6</td>
<td>10/27</td>
<td>0/29</td>
</tr>
<tr>
<td>In vivo</td>
<td>12</td>
<td>38/59</td>
<td>0/60</td>
</tr>
</tbody>
</table>

* Number of antibody-forming cultures/total.
that agglutinin formation was detected in lymph node cultures (14/28) whereas no response could be observed in any of the 38 thymus cultures.

**Antibody Sensitivity to 2-Mercaptoethanol.**—Analysis of the \( \gamma \)-globulin formed

### TABLE V

*Antibody Response in Vitro to SRBC and CHRBC*

<table>
<thead>
<tr>
<th>Spleen No.</th>
<th>Cultures immunized with SRBC</th>
<th>Cultures immunized with CHRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test antigen</td>
<td>SRBC</td>
</tr>
<tr>
<td>1</td>
<td>4/5</td>
<td>0/5</td>
</tr>
<tr>
<td>2</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td>3</td>
<td>4/5</td>
<td>0/5</td>
</tr>
<tr>
<td>4</td>
<td>1/5</td>
<td>0/5</td>
</tr>
<tr>
<td>5</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>6</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td>7</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>8</td>
<td>2/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Number of antibody-forming cultures/total.

### TABLE VI

*Antibody Response to SRBC by Lymph Nodes and Thymus Cultures*

<table>
<thead>
<tr>
<th>Tissue tested</th>
<th>PHA treatment</th>
<th>No. of donors</th>
<th>Incidence of response*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph nodes</td>
<td>In vivo</td>
<td>4</td>
<td>5/8</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>5</td>
<td>9/20</td>
</tr>
<tr>
<td>Thymus</td>
<td>In vivo</td>
<td>4</td>
<td>0/20</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>4</td>
<td>0/18</td>
</tr>
</tbody>
</table>

* Number of antibody-forming cultures/total.

during the antibody response (23, 24) indicated the early formation of \( 19S \) which are mercaptoethanol sensitive, followed by \( 7S \), resistant to the effect of mercaptoethanol. Experiments were therefore performed to test whether antibodies in this in vitro system are of the mercaptoethanol-sensitive or mercaptoethanol-resistant type and whether appearance of antibodies follows the same pattern of response. Forty antibody-forming cultures were tested repeatedly during the first 10 days of the response; all of them exhibited sensitivity to the
Effect of 2-mercaptoethanol. In a similar way, cultures responding with antibodies to CHRBC revealed sensitivity to the effect of mercaptoethanol when tested during the 1st wk. On the 11th day of culture, agglutination persisted following the 2-ME treatment in 6 of 40 cultures, of these, 4 had not been exposed to PHA and the others originated in PHA-injected mice. These cultures continued to exhibit resistance to the effect of the 2-ME subsequently.

**TABLE VII**

*Effect of Puromycin on Antibody Response in Vitro*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>PHA treatment</th>
<th>Type of antibody response induced</th>
<th>Puromycin dosage</th>
<th>Incidence of response*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>In vivo</td>
<td>Primary, induced in vitro</td>
<td>γ</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Primary, induced in vivo 1 day prior to explantation</td>
<td>10</td>
<td>0/39 30/40</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Primary, induced in vivo 5 days prior to explantation</td>
<td>10</td>
<td>5/5 5/5</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Secondary, induced in vitro‡</td>
<td>10</td>
<td>5/5 5/5</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>Primary, induced in vitro</td>
<td>10</td>
<td>0/10 8/10</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>“““</td>
<td>2.5</td>
<td>0/10 8/10</td>
</tr>
</tbody>
</table>

* Number of antibody-forming cultures/total.
‡ Donors injected with SRBC 85 days before cultivation of spleen.

In parallel to this, antibodies from 10 cultures of in vivo hyperimmunized spleens and 10 cultures in which a secondary type of response was induced in vitro were resistant to 2-ME treatment for the entire experimental period.

*Puromycin Effect on Antibody Formation in Vitro.*—Since the presence of natural agglutinins in various strains of mice have been reported as a common phenomenon, one might argue that antibodies appearing in this system have not been induced to be formed in vitro, but rather stimulated to be released. To test whether *de novo* antibody formation is involved in the system or whether a release of already existing antibodies takes place, puromycin was applied to inhibit protein synthesis (25), the assumption being that if antibodies are synthesized in culture their formation might be inhibited in the presence of puromycin, whereas if only a release is involved, their appearance might take place in spite of the puromycin treatment.
Eight spleens were employed in this study, in two repeated experiments. Ten cultures were prepared from each spleen, five of which were incubated in puromycin (10 μg/ml) containing medium. After 5 days, none of the puromycin-treated cultures responded with agglutinins whereas 30 of the 40 control cultures were reacting (Table VII).

To rule out the possibility that puromycin acted primarily by exhibiting a general toxic effect to the tissues under these experimental conditions, control cultures were set up in parallel, in which spleen explants from mice hyperimmunized to SRBC were treated with puromycin. In these, however, antibodies were detectable in all of the 10 cultures treated with puromycin as well as in the untreated controls (Table VII). Since puromycin at the dosage used in this experiment appeared to have, however, some toxic effect as judged by histological studies of the tissues an additional experiment was undertaken using a lower dose (2.5 μg/ml) as compared to that used before. Ten cultures were employed for each experimental group and the results indicated that whereas 8 of the 10 control cultures responded with agglutinins, none of the cultures treated with puromycin showed any antibody response (Table VII). Histological studies of these cultures revealed no obvious toxic effects of the puromycin. While by no means conclusive, the results suggest that appearance of agglutinins in the system is not a result of release of preexisting antibodies.

Effect of Irradiation.—The primary immune response has been well demonstrated as radiosensitive in its initial phase (cf. reference 26). The present experiment was undertaken to test whether or not the response induced in vitro is sensitive to the effect of irradiation, the purpose being to use this feature of the primary antibody response as an additional criterion for the characterization of the response obtained in vitro.

Six spleens were removed 24 hr following total-body irradiation. This time interval has been reported (27) as the most radiosensitive for the primary response process. Thirty cultures were tested as compared to 20 unirradiated controls. Antigen was added to all the cultures and agglutinin response was checked during 10 days. No agglutinin reaction was detected in any of the irradiated spleen explants, whereas 8 of the 20 controls responded on the 5th day. That the irradiation under these experimental conditions was not preventing antibody formation per se was shown by the fact that spleen cultures from mice immunized 1 month previous to exposure did form antibodies (20/20 cases) in a manner similar to that of unirradiated preimmunized controls (10/10 cultures).

This, therefore, indicates that (a) the response induced in vitro is sensitive to the effect of X-irradiation, and (b) such sensitivity is not manifested by spleens from donors immunized before exposure.

Morphological Studies of the Cultures.—It has been reported before (15) that cultures responding with antibodies to antigenic stimulation in vitro continuously manifested the agglutinin reaction in the filter well for various lengths of
periods up to about 2 months. The question was then raised whether such cultures maintained their original structure or whether the response represented the existence of functioning cells regardless of maintenance of the original architecture of the tissue.

In order to follow the morphological behavior of these tissues, cultures were fixed for histology on days 3, 5, 6, 8, 9, 10, 11, 16, 22, 25, 30, and 32 following explantation. Cultures from PHA-treated donors (in vitro: 36 cultures; in vivo: 39 cultures) as well as from untreated donors (33 cultures) were studied in parallel with those explanted from preimmunized mice (27 cultures).

During the first 2 days of culture, cells from the peripheral areas of fragment, in particular red blood cells, gradually separated from the tissue and accumulated on the filter around the explant. This subsequently led to some decrease in the total size of the fragment. Histologically, the tissues contained lymphoid portions (Fig. 1) and were always surrounded by reticulum cells by macrophages, and by small lymphocytes either attached to the surface or loose on the Millipore membrane (Fig. 2). Macrophages and small lymphocytes were often detected in close contact (Figs. 3a, 3b, and 4a). In tissues from previously untreated mice explanted at fragment sizes smaller than 0.5 x 0.7 mm surface area lymphocytes were either scattered with no typical organization or were localized in certain areas possibly representing remains of the original follicles. Explants from PHA-treated or preimmunized donors revealed in general maintenance of lymphoid populations for the entire period of study, yet with no obvious distinction of primary and secondary follicular regions (Fig. 5). Mitotic figures were frequent within the lymphoid cell populations. Plasma cells were detected after the 1st wk in some cultures at the outer region of the follicles and mostly surrounding the tissues, in the filter well (Figs. 4a and 4b, and 6a and 6b).

Usually long term functioning cultures appeared to maintain lymphoid structures. However, there did not seem to be an essential correlation in the opposite direction, i.e. unresponsiveness could not always be attributed directly to poor maintenance of original morphology. Some of the cultures which were stimulated with SRBC and did not manifest an antibody reaction, did not show any obvious morphological difference from those responding.

**DISCUSSION**

The present study extends earlier reported observations (15) on an in vitro system in which antibody response to SRBC was elicited. It has been shown here, that maintenance of the tissues was optimal throughout the experimental period, and that lymphoid elements were preserved in contrast to other culture systems (28) where lymphoid follicles have diminished by the 10th day of culture. The difference in observations may be explained in part by the different oxygen atmospheres used. It has already been shown before that a 95% oxygen
The atmosphere is beneficial to an adequate maintenance of lymphoid cells in organ cultures (29, 30). Furthermore, in the present system even an atmosphere containing 47.5% oxygen proved to be insufficient for this purpose: Cultures under such suboptimal conditions underwent gradual necrosis and manifested a transient response (31).

It has been argued that successful induction of antibody response in vitro might depend on a proper maintenance as well as enabled interactions between various cell types. Cellular interactions have been demonstrated in filter well cultures of lymphoid organs (10-14) as well as of other tissue rudiments (9). In the present system a close contact between macrophages and lymphoid cells was often detected. Whether this morphologically visible contact has any functional meaning (3, 32, 33) has not been established here and needs further critical studies. If indeed interaction between macrophages and lymphocytes is an essential component of antibody-forming process this is probably enabled in the present system.

The assays used for detecting antibodies were filter well agglutination (15) and titrations of the media. One ought to keep in mind, however, that the given titers represent amounts on alternate days following entire discard of remaining medium at the time tested and not total accumulations. Furthermore, these titers represent amounts of antibodies liberated at these time intervals by fragments smaller than 1.0 mm² into a volume of 1.0 ml medium. This represents by itself a considerable dilution. In this context the filter well agglutination is more sensitive, showing the immediate reaction between antibodies liberated by the explant and the adjacent available antigen. This character of the system represents certain advantages over other known techniques: It enables continuous observations on individual cultures with accurate estimations of the onset as well as the end of the response without sacrificing any part of the tissue for the entire period. The need for such a system is of a particular importance in view of the variability in response of different cultures originating even from the same spleen. Thus, kinetic studies can be performed on individual explants, including quantitative as well as qualitative (i.e. mercaptoethanol sensitivity) analysis.

The variability of response manifested by different explants can be attributed to several alternatives among which one might include: (a) morphological differences between the explants; (b) antigenic competition with the medium constituents might possibly lead to varying responses to the antigen in test (34); (c) a heterogeneous distribution of immunocompetent cells which could a priori respond to any given antigen but limited in total number; and (d) a large original number of immunocompetent cells, yet of a heterogeneous precommitment to a wide spectrum of antigens, thus resulting in varying availability of cells competent to the antigen in test. The first suggestion is not supported by the histological observations, showing no direct correlation between unresponsive-
ness and lymphoid appearance of the tissue. The second assumption seems unlikely in view of similar observations reported from in vivo experiments (35) where the number of hemolysin–plaque-forming cells varied in different regions of the same spleen without any introduction of culture medium components at the time of immunization. It is, however, impossible at the present, to determine whether either of the last two alternatives represent a probable explanation. Appropriate cloning or dual antigen experiments might aid in distinguishing between these suggested mechanisms.

It has been argued in a preceding report (15) that the response manifested is of a primary type nature. The argument was based on the specificity of the reaction and on kinetic studies compared to those of primary and secondary responses induced in vivo and maintained in vitro. The present study extends the characterization of the response, demonstrating that a specific antibody response can also be induced to chick erythrocytes. Furthermore, the reaction to SRBC was inhibited by puromycin which had no effect on the response in vitro of previously immunized spleen explants, thus suggesting that antibodies were synthesized de novo rather than liberated from a source of preexisting (“natural”) antibodies. In addition, evidence has been presented that antibodies detected during the first 10 days of response were sensitive to the effect of 2-mercaptoethanol, and that subsequently some of the cultures elicited antibodies which were resistant. Similar sequential changes have been demonstrated before in vivo antibody-forming systems (23, 24) and recently reported in in vitro response to φX-174 phage antigen (16). One might still argue that the reaction in vitro was enabled by a possible preexistence of genetic information within the immunocompetent cells to respond to the given antigen without any overt evidence for the presence of “natural” antibodies. This question in fact, is a most fundamental one being as yet an unsolved problem in the mechanism of antibody formation even in the in vivo situations. Consequently, as long as immune reactions elicited in vitro manifest the same characteristics as those designated in in vivo systems as “primary-response” there seems to be no adequate reason why not to include the presently described reaction in the same category.

SUMMARY

Specific antibody formation has been elicited in vitro following antigenic stimulation by either sheep (in a total of 472 of 875 cultures) or chick erythrocytes (in 65 of 135 cultures tested). The response was manifested by mouse spleen and lymph node explants whereas thymus cultures were inactive. The reaction has been characterized as a primary immune response in view of its kinetics as compared to defined primary and secondary responses, the effect of 2-mercaptoethanol on the antibodies formed, its subject to puromycin inhibition and its sensitivity to X-irradiation. Histological studies revealed preservation of the lymphoid cell populations throughout the entire experimental period.
The excellent technical assistance of Mrs. Tehila Umiel, Miss Barbara Alter, Mr. Louis Kubai, and Mr. Neil Halsey is greatly acknowledged.

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**EXPLANATION OF PLATES**

**PLATE 93**

Fig. 1. Spleen culture, explanted from PHA-injected donor, agglutinin response was detected, 5 days of culture. × 155.

Fig. 2. Spleen culture explanted from an untreated donor, agglutinin response was detected, 3 days of culture. × 398.
(Globerson and Auerbach: Primary antibody response in vitro)
PLATE 94

Figs. 3 a and 3 b. 8-day-old culture originating in an untreated donor. Macrophages and lymphocytes are shown (Fig. 3 a) detached from the explant or (Fig. 3 b) at the immediate surface area. × 1550.

Fig. 4 a. Spleen explanted from PHA-treated donor, 22 days of culture. Lymphocytes are apparent within the explant, whereas reticulum cells, lymphocytes, and plasma cells are visible around the surface area of tissue. × 631.

Fig. 4 b. Plasma cells detached from the explant, 25 days of spleen culture originating from a PHA-treated donor. × 1550.
(Globerson and Auerbach: Primary antibody response in vitro)
PLATE 95

Fig. 5. 25-day-old spleen culture, originating from a PHA-treated donor. × 398.

Figs. 6a and 6b. Plasma cells at the outer surface area of spleen explant, originating from a PHA-treated donor. 25 days of culture. × 631.
(Globerson and Auerbach: Primary antibody response in vitro)