SPECIFIC HETEROLOGOUS ENHANCEMENT OF IMMUNE RESPONSES

IV. SPECIFIC GENERATION OF A THYMUS-DERIVED ENHANCING FACTOR*

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Recent experimental evidence has provided strong support for the concept that at least two distinct populations of lymphoid cells cooperate in the production of humoral antibody. Synergism between thymus-derived (T cell) and bone marrow-derived (B cell) lymphocytes has been described in the induction of antibody synthesis against heterologous erythrocytes (1–3), serum proteins (4, 5), and hapten-protein conjugates (6–8). Although both participating cell types can respond to specific antigenic stimulation (9, 10), actual synthesis of exportable immunoglobulin is apparently limited to cells of B lymphocyte lineage (11, 12). While T cells therefore do not secrete detectable amounts of antibody, they appear in some cases to facilitate antibody production by B cells. However, the mechanism of interaction between these components is not well defined.

We recently demonstrated that augmented immune responses similar to those after reexposure to the same antigen need not be specific (13). Inbred mice were primed with tetanus toxoid (TT); later, their spleen cells were maintained in culture with sheep erythrocytes (SRBC). The addition of TT (the antigen used initially for priming) to these cultures resulted in significant enhancement of the anti-SRBC plaque-forming cell (PFC) response as compared to aliquots cultured without TT. The addition of TT to comparable cultures of spleen cells from normal mice, or the addition of another antigen to TT-primed cultures, failed to produce enhancement of anti-SRBC plaques. Moreover, augmentation of the number of plaques was obtained by adding TT to mixed cultures of normal spleen cells plus thymocytes from TT-primed mice (14). These observations were consistent with the concept, first suggested by Davies et al. (11), that a mediator released from specifically stimulated T cells acts on the antibody-forming B cells to increase their productive response. Supernatants derived from

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Abbreviations used in this paper: B cell, bone marrow-derived lymphocyte; T cell, thymus-derived lymphocyte; PFC, plaque-forming cell; SRBC, sheep erythrocytes; TT, fluid tetanus toxoid; FBS, fetal bovine serum; EF, enhancing factor; ThyT+, supernatants from primed thymocytes cultured with tetanus toxoid; ThyT-, supernatants from primed thymocytes cultured without tetanus toxoid; SplTS+, supernatants from primed spleen cells cultured with tetanus and sheep erythrocytes; SplT+, supernatants from primed spleen cells cultured with tetanus toxoid; Thy, thymocytes; Spl, spleen cells.
cultures of tetanus toxoid stimulated, TT-primed spleen cells were tested for such an
effector molecule. We found that the cell-free culture fluid, when added to SRBC-
stimulated, normal spleen cell cultures, significantly enhanced the anti-SRBC PFC
response (15). This activity was termed enhancing factor (EF).

The purpose of this paper was to confirm and extend the finding that a
humoral factor, released upon specific interaction of antigen with a primed
population of T cells, can increase the antibody response to another antigen
present in the system. In cell cultures containing SRBC-stimulated normal
spleen cells, and TT-primed cells from either thymus or spleen, enhancement
of the anti-SRBC PFC response occurred when 1 ng of TT was added, although
tenfold fewer thymocytes than spleen cells were needed to produce comparable
results. Furthermore, supernatants of cultures of thymocytes from TT-primed
mice and TT were found to contain a soluble enhancing factor. It was absent
from similar cultures of normal thymocytes plus TT, and from primed thymo-
cytes cultured without TT.

Materials and Methods

Immunization of Mice.—8-wk old DBA/2 female mice (Jackson Laboratory, Bar Harbor,
Maine) were immunized intravenously with 10 Lf of fluid tetanus toxoid (Massachusetts
Department of Public Health, Division of Biologic Laboratories, Boston, Mass.). Mice were
shown to be primed by finding antitoxin in their serum, as previously described (13).

Preparation of Spleen Cell Cultures.—Cell
cultures were prepared from the spleens of im-
umunized and normal mice 30–60 days after injection by the method of Mishell and Dutton
(16) as modified by Mosier (17). Each culture contained 1 ml of cells (2 X 10^7) suspended in
medium supplemented with 10% fetal bovine serum (FBS) (Reheis Chemical Co., Kankakee,
Ill.). Cultures were fed daily with a nutritional supplement (13) and were stimulated with
approximately 3 million SRBC on the day of culture (day 0). 1 ng of tetanus toxoid was added
to half of these cultures on day 2. The in vitro dose of TT and the time of addition were
needed to elicit the optimal enhancement effect (13). Some cultures received only the priming
antigen in the absence of SRBC stimulation.

Preparation of Mixed Lymphoid Cultures.—Suspensions of TT-primed thymocytes and
normal thymocytes were prepared as described previously (14). Briefly, the two-thirds of
the thymic lobes distal to the hilus were excised, care being taken to avoid fascial tags which might
contain lymph node tissue. All adherent connective tissue was removed. A number (approxi-
mately 10^6) of the DBA/2 mice used as thymus donors were injected intraperitoneally with
0.2 ml of Pelikan ink (Günther Wagner, Hanover, Germany; lot No. C11/1431A) diluted 1:1
with saline, in order to reveal any parathyroid nodes as found by Leckband and Boyse (18).
None were found in DBA/2 mice. In contrast, in C3H mice treated similarly, parathyroid
lymph nodes were easily detected in the connective tissue lateral to the thymic hilus. Thymus
lobes were teased apart and treated similarly to the spleen cell suspensions. Thymocytes were
suspended in complete medium (13) to a final concentration of 20 million cells/ml. Experimental
cultures contained 10 million normal spleen cells and 10, 1, or 0.1 million primed or
normal thymocytes. Parallel mixed cultures containing spleen cells from both immunized and
normal mice were also prepared. Mixed lymphoid cell cultures were stimulated with TT and
SRBC as described above and direct "19S" plaques enumerated on day 5 by a modification of
the method of Jerne and Nordin (13).

Preparation of Supernatants.—On day 5, the spleen cell culture fluid was collected and
centrifuged to remove cells (200 g, 10 min, 4°C). Supernatants harvested from three to five
cultures per experimental group were stored at \(-20^\circ\text{C}\) until assayed for enhancing activity. In addition, supernatants were prepared from short-term cultures of thymocytes from TT-primed and normal animals by the methods described above. Thymocyte cultures containing 20 million cells were stimulated with 1 ng of TT on day 0. Half of the cultures were unstimulated. Supernatants were collected sequentially from replicate cultures at 24, 48, and 72 hr and were treated as described earlier.

**Evaluation of Supernatant Activity.**—Supernatants from cultures of spleen cells or thymocytes were assayed for the ability to augment the anti-SRBC PFC response by methods previously described (15). Briefly, spleen cell cultures from normal mice were prepared and were stimulated with 3 million SRBC on day 0. Filtered supernatants, diluted in fresh medium, were added to such cultures on day 2. Direct plaques were counted on day 5.

The degree of enhancement is expressed as the per cent difference between the numbers of plaques produced in cultures that received test supernatants, compared to the PFC response of cultures that received the appropriate dilution of a normal control supernatant, i.e., derived from normal spleen (or normal thymocyte) cultures not receiving antigen. Normal control supernatants were used as base line controls to correct for any enhancing activity provided by FBS, by constituents released from dying cells, or produced by viable cells in response to FBS foreign antigenic determinants. Supernatants assayed for enhancing factor included those derived from TT-primed spleen cell cultures that received (a) both tetanus and SRBC (SplTS\(^+\)), or (b) tetanus toxoid alone (SplT\(^+\)). Supernatants from cultures of TT-primed thymocytes maintained in the presence or absence of toxoid were also tested for enhancing factor. These supernatants were designated ThyT\(^+\) and ThyT\(^-\), respectively. Comparable supernatants from cultures of unprimed, normal thymocytes were tested in parallel.

**Heat Treatments.**—ThyT\(^+\) supernatants were tested for the stability of enhancing factor to mild heat treatment. One-half of each supernatant was heated at 56°C for 30 min. Each aliquot was then diluted and the activity assessed as described above.

**Dialysis.**—ThyT\(^+\) supernatants were divided into two aliquots. One aliquot was dialyzed against 1000 volumes of isotonic saline for 24 hr at 4°C; an equal volume was kept cold, but not dialyzed. Treated and untreated aliquots were added to assay cultures and tested for the ability to amplify the anti-SRBC PFC response.

**Enzyme Treatments.**—Supernatants with enhancing activity (SplTS\(^+\), SplT\(^+\), and ThyT\(^+\)) were tested for their sensitivity to enzymatic degradation with deoxyribonuclease I (DNAse, lot No. 5613; Worthington Biochemical Corp., Freehold, N. J.), ribonuclease A (RNAse, lot. No. 100C-8062; Sigma Chemical Co., St. Louis, Mo.), or protease (type VI, lot No. 71C-9020; Sigma Chemical Co.). Supernatants were divided into two aliquots. DNAse, RNAse, or protease was added to one aliquot at a final concentration of 10 \(\mu\text{g}/\text{ml}\). Supernatant-enzyme mixtures were mixed constantly (1 hr, 37°C) to ensure adequate contact between enzyme and substrate. Treated and untreated aliquots were then assayed for activity by the usual method.

**RESULTS**

**Relative Numbers of Primed Thymocytes or Spleen Cells Needed to Produce Enhancement in Mixed Cell Cultures.**—In a previous paper we reported that at least some components of antigen-specific immunological memory reside in the thymus of mice immunized 1–2 months earlier (14). Specific stimulation in vitro of thymocytes from TT-primed mice, cultured with SRBC-stimulated normal spleen cells, produced a significant increase in the anti-SRBC hemolysin response as measured by plaque formation (14). In order to quantitate the number of thymocytes necessary to produce this amplifying effect in vitro, cell mixtures containing 10 million normal spleen cells and graded numbers of
syngeneic thymocytes from mice immunized with TT were stimulated with SRBC and the specific priming antigen. A parallel study was performed substituting TT-primed spleen cells for the TT-primed thymocytes. The results of a representative group of experiments are shown in Table I. The addition of 1 ng of TT on day 2 to SRBC-stimulated, normal spleen cell cultures containing 10 million or 1 million TT-primed thymocytes produced an average of 67% and 89% enhancement, respectively, of the anti-SRBC PFC response compared with similar cultures without the priming antigen (groups 1, 2 and 5, 6). The addition of TT to parallel cultures containing similar numbers of normal spleen cells and normal thymocytes resulted in a 20% and 18% depression, respectively, in the anti-SRBC response (groups 3, 4 and 7, 8). Although 1 million primed thymocytes could be triggered in vitro to produce nonspecific enhancement of the PFC response, when the number of primed thymocytes was reduced to 1 X 10^6 per culture, the addition of TT failed to elicit an augmented PFC response (groups 9 and 10). Similar additions of priming antigen to positive control (groups 13 and 14) and negative control cultures (groups 15 and 16), from representative experiments, produced augmentation or slight depression, respectively, of the PFC response, as previously reported (13). Cultured thymocytes alone, stimulated with TT and SRBC, failed to produce any plaques (13). In parallel experiments, in which 10 million TT-primed spleen cells were substituted for the 10 million primed thymocytes, the addition of 1 ng of toxoid resulted in a degree of augmentation of the anti-SRBC PFC response comparable to that seen in Table I (groups 1 and 2, Table II). However, in contrast to the enhancement seen in mixtures of 1 million primed thymocytes and 10 million normal spleen cells, the addition of priming antigen to cultures with similar numbers of primed spleen cells and normal spleen cells resulted in a 14% depression in the anti-SRBC response (groups 5 and 6, Table II). Enhancement was consistently observed in representative positive controls, (groups 9 and 10), while slight depression was seen in the negative controls (groups 11 and 12).

Release of Enhancing Factor from Thymocytes.—From the data in Table I it is apparent that the addition of the specific priming antigen to primed thymocytes maintained in the presence of normal spleen cells resulted in the non-specific augmentation of the in vitro immune response to a non-cross-reacting (13) antigen. It was of interest to determine whether the mechanism of this enhancement phenomenon was the same as that previously reported for specifically stimulated spleen cells (15), i.e., a humoral factor released by the specific interaction of antigen with a primed population of T cells can increase the productive antibody response of B cell lymphocytes reacting to a heterologous antigen. Since thymocytes maintained in the absence of a supporting lymphoid cell population do not survive for extended periods of time in vitro (19), we cultured TT-primed and normal T cells from the thymus for 24–72 hr.

2 Rubin, A. S. Unpublished observations.
TABLE I

Effect on the Anti-SRBC PFC Response of Adding 1 ng of TT on Day 2 to Mixed Cultures of 10,
1, or 0.1 Million TT-Primed Thymocytes and 10 Million Normal Spleen Cells
Stimulated with SRBC on Day 0

<table>
<thead>
<tr>
<th>Group</th>
<th>Cultured cells (in millions)</th>
<th>Anti-SRBC plaques per $10^6$ recovered cells</th>
<th>Average enhancement of anti-SRBC PFC response</th>
<th>$p^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>Experiment</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10 Primed Thy 10 Normal Spl</td>
<td>+ 250</td>
<td>1793</td>
<td>1530</td>
</tr>
<tr>
<td>2</td>
<td>10 Primed Thy 10 Normal Spl</td>
<td>- 161</td>
<td>883</td>
<td>913</td>
</tr>
<tr>
<td>3</td>
<td>10 Normal Thy 10 Normal Spl</td>
<td>+ 172</td>
<td>1623</td>
<td>1380</td>
</tr>
<tr>
<td>4</td>
<td>10 Normal Thy 10 Normal Spl</td>
<td>- 261</td>
<td>1617</td>
<td>1633</td>
</tr>
<tr>
<td>5</td>
<td>1 Primed Thy 10 Normal Spl</td>
<td>+ 1153</td>
<td>1527</td>
<td>973</td>
</tr>
<tr>
<td>6</td>
<td>1 Primed Thy 10 Normal Spl</td>
<td>- 657</td>
<td>603</td>
<td>710</td>
</tr>
<tr>
<td>7</td>
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<td>+ 810</td>
<td>1383</td>
<td>853</td>
</tr>
<tr>
<td>8</td>
<td>1 Normal Thy 10 Normal Spl</td>
<td>- 983</td>
<td>1910</td>
<td>1267</td>
</tr>
<tr>
<td>9</td>
<td>0.1 Primed Thy 10 Normal Spl</td>
<td>+ 2027</td>
<td>1173</td>
<td>2060</td>
</tr>
<tr>
<td>10</td>
<td>0.1 Primed Thy 10 Normal Spl</td>
<td>- 2173</td>
<td>1227</td>
<td>2367</td>
</tr>
<tr>
<td>11</td>
<td>0.1 Normal Thy 10 Normal Spl</td>
<td>+ 2307</td>
<td>947</td>
<td>2567</td>
</tr>
<tr>
<td>12</td>
<td>0.1 Normal Thy 10 Normal Spl</td>
<td>- 2500</td>
<td>1433</td>
<td>2720</td>
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<td>3347</td>
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<td>14</td>
<td>20 Primed Spl 20 Normal Spl</td>
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<td>2140</td>
<td>2050</td>
</tr>
<tr>
<td>15</td>
<td>20 Normal Spl 20 Normal Spl</td>
<td>+ 660</td>
<td>2587</td>
<td>2173</td>
</tr>
<tr>
<td>16</td>
<td>20 Normal Spl 20 Normal Spl</td>
<td>- 726</td>
<td>2660</td>
<td>2587</td>
</tr>
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</table>

Nos. in parentheses indicate degree of enhancement in per cent.

* Calculated by Student's $t$ test to determine the significance of the difference between the mean enhancement produced in TT-primed cultures and the mean increase produced by the corresponding, similarly-treated, normal cultures.
Specific enhancement of immune responses

Table II
Effect on the Anti-SRBC PFC Response of Adding 1 ng of TT on Day 2 to Mixed Cultures of 10 Million (or 1 Million) TT-Primed Spleen Cells and 10 Million Normal Spleen Cells Stimulated with SRBC on Day 0

<table>
<thead>
<tr>
<th>Group</th>
<th>Cultured cells (in millions)</th>
<th>Anti-SRBC plaques per 10^6 recovered cells</th>
<th>Average enhancement of anti-SRBC PFC response</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>10 Primed Spl</td>
<td>+</td>
<td>2620</td>
<td>2480</td>
</tr>
<tr>
<td></td>
<td>10 Normal Spl</td>
<td></td>
<td>(61)</td>
<td>(89)</td>
</tr>
<tr>
<td>2</td>
<td>10 Primed Spl</td>
<td>−</td>
<td>1627</td>
<td>1313</td>
</tr>
<tr>
<td></td>
<td>10 Normal Spl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10 Normal Spl</td>
<td>+</td>
<td>1547</td>
<td>1833</td>
</tr>
<tr>
<td></td>
<td>10 Normal Spl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10 Normal Spl</td>
<td>−</td>
<td>2687</td>
<td>1813</td>
</tr>
<tr>
<td></td>
<td>10 Normal Spl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1 Primed Spl</td>
<td>+</td>
<td>867</td>
<td>1333</td>
</tr>
<tr>
<td></td>
<td>10 Normal Spl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1 Primed Spl</td>
<td>−</td>
<td>1060</td>
<td>2053</td>
</tr>
<tr>
<td></td>
<td>10 Normal Spl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1 Normal Spl</td>
<td>+</td>
<td>1040</td>
<td>1807</td>
</tr>
<tr>
<td></td>
<td>10 Normal Spl</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>1 Normal Spl</td>
<td>−</td>
<td>1487</td>
<td>1867</td>
</tr>
<tr>
<td></td>
<td>10 Normal Spl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>20 Primed Spl</td>
<td>+</td>
<td>1740</td>
<td>1840</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(50)</td>
<td>(71)</td>
</tr>
<tr>
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<td>20 Primed Spl</td>
<td>−</td>
<td>1160</td>
<td>1073</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>20 Normal Spl</td>
<td>+</td>
<td>900</td>
<td>1380</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td>(-12)</td>
</tr>
<tr>
<td>12</td>
<td>20 Normal Spl</td>
<td>−</td>
<td>1120</td>
<td>1567</td>
</tr>
</tbody>
</table>

See Table I for explanatory footnotes.

1 ng of TT was added at day 0. Cell-free supernatants were harvested at daily intervals and were assayed for biological activity. Table III is a typical group of experiments which show the reproducible effect on the anti-SRBC PFC response of adding 24 hr supernatants on day 2 to normal spleen cell cultures that received SRBC on day 0. Addition of Thy+ supernatants derived from specifically-stimulated primed thymocytes to assay cultures resulted in a
TABLE III

Effect on the Anti-SRBC PFC Response of Adding 24-hr Thymocyte Culture Supernatants on Day 2 to Normal Spleen Cell Cultures That Received SRBC on Day 0

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Origin</th>
<th>Antigens added to culture</th>
<th>Final dilution of supernatant</th>
<th>Anti-SRBC plaques per 10^6 recovered cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
<td>1:30</td>
</tr>
<tr>
<td>1</td>
<td>TT-primed</td>
<td>TT</td>
<td>1447 (-21)</td>
<td>1453 (-22)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>TT</td>
<td>2112 (+15)</td>
<td>1480 (-20)</td>
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<td></td>
<td>Normal</td>
<td>None</td>
<td>1840</td>
<td>1860</td>
</tr>
<tr>
<td>2</td>
<td>TT-primed</td>
<td>TT</td>
<td>1327 (+7)</td>
<td>1247 (-5)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>TT</td>
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</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td>1240</td>
<td>1307</td>
</tr>
<tr>
<td>3</td>
<td>TT-primed</td>
<td>TT</td>
<td>1687 (-14)</td>
<td>2027 (+6)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>TT</td>
<td>1887 (-4)</td>
<td>1873 (-2)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td>1973</td>
<td>1920</td>
</tr>
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</table>

* Calculated by Student's t test to determine the significance of the difference between the mean enhancement produced by the optimum dilutions of supernatants from TT-primed cultures and the mean increase produced by the corresponding dilutions of normal supernatants.

† Numbers in parentheses indicate the degree of enhancement (in per cent) compared with the PFC response of normal cultures that had received supernatant from normal, unstimulated cultures.

significant increase in the anti-SRBC plaque response compared with similar cultures that received normal control supernatants. The average increase obtained, with a 1:100 dilution of the active supernatant, was 47% (P < 0.005). No such increase was found with culture medium from TT-stimulated normal thymocytes (Table III) or from unstimulated primed thymocytes (Fig. 1). Individual supernatants contained varying amounts of enhancing activity since optimal augmentation of the hemolytic plaque response was obtained with either 1:30 or 1:100 dilutions of the culture fluid (Tables III–VI). Dilutions other than the optimal dilution generally depressed the PFC response or enhanced it to an insignificant degree.

Kinetics of Release of Enhancing Factor.—Fig. 1 shows the results of a representative experiment in which supernatants from TT-primed thymocytes, maintained in the presence or absence of toxoid, were harvested from replicate cultures at 24-hr intervals and tested for enhancing activity. It can be seen that virtually all of the enhancing material was released from TT-stimulated primed thymocytes by 48 hr of culture, although a significant amount of activity was present in the 24-hr supernatants (Table III). The anti-SRBC PFC response
was not significantly increased by culture fluid from TT-primed thymocytes maintained without TT (Fig. 1, lower curve).

**Effect of Heat Treatment on Activity of Thymocyte-Derived Enhancing Factor.**—Aliquots of ThyT+ supernatants, harvested at 48 hr, were heated at 56°C for 30 min and tested for the ability to amplify the production of anti-SRBC plaques in assay cultures compared to the enhancing activity of similar, unheated aliquots. Table IV (experiments 1 and 2) demonstrates that the activity present in the ThyT+ supernatants was unaffected by mild heat treatment. Diluted aliquots of heated and unheated supernatants produced comparable degrees of enhancement of the PFC response when added on day 2 to normal spleen cell cultures that received SRBC on day 0.

**Effect of Dialysis on Activity of Thymocyte-Derived Enhancing Factor.**—Supernatants from 48-hr cultures of TT-primed, TT-stimulated thymocytes were divided into aliquots. One aliquot was dialyzed against 1000 volumes of isotonic saline for 24 hr at 4°C, while the other was kept cold, but not dialyzed. The results from two independent experiments are shown in Table IV (experiments 3 and 4). It can be seen that the dialyzed and undialyzed supernatants were equally effective in amplifying the anti-SRBC PFC response of the assay cultures.

**Effect of DNase, RNAse, and Protease on Enhancing Factor.**—Culture fluids from TT-stimulated spleen cells or thymocytes were divided and treated with one of three enzymes (DNase I, RNAse A, or protease) in an attempt to define the chemical nature of enhancing factor. One aliquot of each supernatant was mixed with 10 μg/ml of enzyme for 1 hr at 37°C, while a similar aliquot was left untreated. Tables V and VI demonstrate that the activity of enhancing...
### TABLE IV

**Effect on the Anti-SRBC PFC Response of Adding Heat-Treated or Dialyzed 48-hr Culture Supernatants on Day 2 to Normal Spleen Cell Cultures That Received SRBC on Day 0**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Origin</th>
<th>Antigen added to culture</th>
<th>Supernatant treatment</th>
<th>Assay culture of normal spleen cells to which supernatant was added</th>
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</thead>
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<td></td>
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<td></td>
<td>Anti-SRBC plaques per 10^6 recovered cells</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>Final dilution of supernatant*</td>
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<td></td>
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<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>1720 (-19)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT</td>
<td>Heat*</td>
<td>2020 (-5)</td>
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<td>Normal</td>
<td>None</td>
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</tr>
<tr>
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<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>2087 (-1)</td>
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<tr>
<td></td>
<td>TT-primed</td>
<td>TT</td>
<td>Heat*</td>
<td>2127 (+1)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td></td>
<td>2100</td>
</tr>
<tr>
<td>3</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>2047 (+1)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT</td>
<td>Dialysis‡</td>
<td>2433 (+20)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td></td>
<td>2033</td>
</tr>
<tr>
<td>4</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>1780 (+2)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT</td>
<td>Dialysis‡</td>
<td>1967 (+12)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td></td>
<td>1753</td>
</tr>
</tbody>
</table>

See Table III for explanatory footnotes.

* Supernatants heated at 56°C, 30 min.
‡ Supernatants dialyzed against 1000 volumes of normal, isotonic saline for 24 hr at 4°C.

The factor was not significantly affected by treatment with DNase or RNase, respectively, whether EF was derived from spleen or thymocyte culture medium. However, exposure of active supernatants to protease resulted in a significant decrease in their ability to augment the anti-SRBC response of normal spleen cells (Tables V and VI). As a control for possible toxicity of protease to the assay cultures, 1 μg of the enzyme, the maximum amount added with diluted supernatants to test cultures, was added to SRBC-stimulated spleen cell cultures. Similar numbers of plaques were obtained in cultures maintained with or without this amount of protease. Thus, the reduction of enhancing activity was due to enzymatic degradation of the factor, and not due to toxicity.

### DISCUSSION

It is now clearly recognized that thymus-derived lymphocytes play an important functional role in facilitating the humoral immune response of B cells to a variety of antigens (10, 20, 21). But the actual mechanism of the
### TABLE V

**Effect on the Anti-SRBC PFC Response of Adding Enzyme-Treated, Spleen Cell Culture Supernatants on Day 2 to Normal Spleen Cell Cultures that Received SRBC on Day 0**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Origin</th>
<th>Antigen added to culture</th>
<th>Supernatant treatment*</th>
<th>Anti-SRBC plaques per 10^6 recovered cells</th>
<th>Final dilution of supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
</tr>
<tr>
<td>1</td>
<td>TT-primed</td>
<td>TT, SRBC</td>
<td>None</td>
<td>527 (-42)</td>
<td>735 (-40)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT, SRBC</td>
<td>DNAse</td>
<td>633 (-6)</td>
<td>770 (-37)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
<td>910</td>
<td>1233</td>
</tr>
<tr>
<td>2</td>
<td>TT-primed</td>
<td>TT, SRBC</td>
<td>None</td>
<td>1243 (-11)</td>
<td>1190 (-8)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT, SRBC</td>
<td>DNAse</td>
<td>1490 (+2)</td>
<td>1470 (+19)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
<td>1397</td>
<td>1233</td>
</tr>
<tr>
<td>3</td>
<td>TT-primed</td>
<td>TT, SRBC</td>
<td>None</td>
<td>2113 (-1)</td>
<td>3660 (+46)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT, SRBC</td>
<td>RNAse</td>
<td>2800 (+14)</td>
<td>3110 (+31)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
<td>2447</td>
<td>2373</td>
</tr>
<tr>
<td>4</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>1753 (-28)</td>
<td>2540 (+9)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT</td>
<td>RNAse</td>
<td>1827 (-25)</td>
<td>2939 (+7)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
<td>2127</td>
<td>2127</td>
</tr>
<tr>
<td>5</td>
<td>TT-primed</td>
<td>TT, SRBC</td>
<td>None</td>
<td>1360 (-40)</td>
<td>2967 (-17)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT, SRBC</td>
<td>Protease</td>
<td>1407 (-46)</td>
<td>2247 (-10)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
<td>2620</td>
<td>2493</td>
</tr>
<tr>
<td>6</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>1547 (-11)</td>
<td>2093 (-26)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT</td>
<td>Protease</td>
<td>2920 (+11)</td>
<td>3047 (+7)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
<td>2627</td>
<td>2847</td>
</tr>
<tr>
<td>7</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>1447 (-35)</td>
<td>1833 (-17)</td>
</tr>
<tr>
<td></td>
<td>TT-primed</td>
<td>TT</td>
<td>Protease</td>
<td>2060 (+7)</td>
<td>1753 (-22)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td></td>
<td></td>
<td>1927</td>
<td>2240</td>
</tr>
</tbody>
</table>

* Supernatants treated with 10 µg/ml of enzyme, 1 hr, 37°C. See Table III for explanatory footnotes.

"helper cell" activity is not yet understood. A number of alternative experimental models have been proposed to explain the mechanism of T cell-B cell interaction (22, 23). These hypotheses were summarized by Dutton et al. (23) as follows. (a) The T cell concentrates antigen on its surface, presumably through binding to specific receptor molecules, and presents multiple determinants to the B cell, forming an antigen-receptor bridge between the two lymphocytes. (b) Surface membrane interaction between T and B cells, in addition to antigen-bridging between the cells, is needed. (c) The B cell must receive two signals: one from its own receptor via antigen-binding and a second signal from the "carrier antibody" receptor on, or released by, the T cell. (d) Specifically activated T cells release a nonspecific chemical mediator which triggers B cells that have already bound antigen through receptors on their
### TABLE VI
Effect on the Anti-SRBC PFC Response of Adding Enzyme-Treated, Thymocyte Culture Supernatants on Day 2 to Normal Spleen Cell Cultures That Received SRBC on Day 0

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Origin</th>
<th>Antigens added to culture</th>
<th>Supernatant treatment*</th>
<th>Assay culture of normal spleen cells to which supernatant was added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Anti-SRBC plaques per 10^6 recovered cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
</tr>
<tr>
<td>1</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>913 (-16)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td>DNAse</td>
<td>913 (-16)</td>
</tr>
<tr>
<td>2</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>2607 (-10)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td>DNAse</td>
<td>3020 (+4)</td>
</tr>
<tr>
<td>3</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>1473 (-21)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td>RNAse</td>
<td>1987 (+6)</td>
</tr>
<tr>
<td>4</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>2310 (-18)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td>RNAse</td>
<td>2560 (-10)</td>
</tr>
<tr>
<td>5</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>1667 (-44)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td>Protease</td>
<td>1487 (-50)</td>
</tr>
<tr>
<td>6</td>
<td>TT-primed</td>
<td>TT</td>
<td>None</td>
<td>2087 (+2)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>None</td>
<td>Protease</td>
<td>1300 (-34)</td>
</tr>
</tbody>
</table>

* Supernatants treated with 10 μg/ml of enzyme, 1 hr, 37°C. See Table III for explanatory footnotes.

Surface membranes. The result of such stimulation of B cells is the production of immunoglobulin molecules which carry the same specificity as that of the receptor for antigen on the B cell surface. An accessory cell, possibly the macrophage, may also play an essential role in T cell–B cell interactions (22, 24, 25). Our data strongly supports model d, although the other postulated mechanisms of collaboration between T and B lymphocytes cannot be totally excluded. We have demonstrated that an enhancing factor (EF) is specifically released from presumably sensitized lymphoid cells by interaction with priming antigen. Once elicited, it can augment the immune response to another, unrelated, antigen. The enhancing activity was generated in vitro by the addition
of the specific antigen used for priming to cultures of spleen cells or thymocytes
derived from the immunized mice. A similar addition of priming antigen to
cultures of normal cells, or cells from mice primed with a heterologous im-
munogen (15), failed to evoke the release of EF. The EF's found in the supern-
atant medium of cultures of either spleen cells, or thymocytes had similar
physicochemical characteristics. The activity of the mediator was nondialyzable
and unaffected by mild heat treatment (56°C, 30 min) or treatment with
DNase or RNase. However, it was inactivated by exposure to protease. Al-
though the factor was not destroyed by pancreatic RNase or DNase, this
does not necessarily exclude the possibility that it can be inactivated by en-
zymes with other specificities or with RNase A or DNase I under different
experimental conditions. We conclude, however, that the soluble factor is
protein in nature.

The fact that an enhancing factor which can affect the productive response
of B cells is released specifically from primed T cells diminishes the need to
postulate actual antigen-bridging between the two classes of cells as a prior
requirement for activation of the B cell component (models a and b). With a
diffusible, soluble effector molecule, close proximity between the cells would
be sufficient. Alternatively, the mediator that we have described could be a T
cell receptor (or carrier antibody), possibly complexed with specific antigen.
When bound to a receptor for antibody (26) or for antigen-antibody complexes
(27) found on the B cell surface, it might induce antibody synthesis in those B
cells that have also bound another antigen by specific antigen-receptor mole-
cules. This double-hit hypothesis is a variation of model c described above.
However, the fact that active supernatants were effective in augmenting the
anti-SRBC PFC response when added to assay cultures on day 2, but not when
added on day 0 of a 5-day culture period (15), may argue against this latter
hypothesis. By day 3, substantial numbers of PFC are present in the spleen
cell cultures (16). Thus, it is not inconceivable that most of the B cells which
can be affected by EF are already past the stage of differentiation at which
they could be stimulated sequentially by antigen and a complex. Moreover,
cellular interactions, both in vitro (28-30) and in vivo (31, 32), between mixed
populations of histoincompatible lymphoid cells produce nonspecific augmenta-
tion of immune responses similar to those we have described; indeed, it is likely
that all these phenomena have a common mechanism which is associated with
blast transformation. In fact, soluble factors produced by T cells in allogeneic
cell mixtures have been reported (33, 34). Thus, if mere contact between
allogeneic cells is sufficient to trigger an elevated, nonspecific response, it is
unlikely that an antigen-antibody complex, or an anti-carrier antibody derived
from T cells, is involved in these cell-to-cell interactions. Furthermore, a dis-
crete, adjuvant-like molecule, produced under a variety of clinical and exper-
imental conditions, could explain many of the nonspecific anamnestic responses
reported over the years (13). Other possible mechanisms of action of EF have
been discussed previously (15).
A number of nonantibody mediators of cellular immunity have been reported in recent years (35). These soluble factors, which mediate in vitro phenomena correlated with delayed hypersensitivity observed in vivo, nonspecifically amplify the activities of a small number of specifically stimulated lymphocytes. Effector molecules which apparently can regulate the humoral immune response have also been described (33, 34, 36–38). Feldmann and Basten (38) have demonstrated that carrier-primed T cells, stimulated with specific hapten-carrier complex, could collaborate with similarly stimulated B lymphocytes to produce a response to the hapten (DNP), although the lymphocytes were physically separated by a cell-impermeable membrane. B cells were unable to respond to the complex in the absence of carrier-stimulated T cells. However, under similar conditions, the factor released specifically from carrier-primed T cells did not augment the immune response to donkey erythrocytes. Therefore, this factor may differ from EF since it only selectively enhanced the response of B cells to specific determinants linked to the specificities recognized by T cells.

Two T cell-derived factors which restored the immune response of T cell-depleted spleen cells to SRBC have recently been described (36, 37). That found by Doria and his colleagues was released from thymocytes cultured with or without antigen (36). Another such material, reported by Gorczynski et al., was nondialyzable and was released by incubation with antigen (37). This latter material is very similar to the substance we have described. Schimpl and Wecker (33) and Britton (34) also found soluble factors which nonspecifically augmented the anti-SRBC PFC response but which were elicited by mixing T cells with lymphoid cells of a different H-2 type. These T cell-replacing factors and EF may have similar characteristics. However, the mediator of Britton (34) can only be found in the supernatants from 24-hr cultures of allogeneic cells, not in media from 48- to 120-hr cultures. Since EF persists for at least 72 hr (Fig. 1), there is a difference in the stability of the two effector molecules. Alternatively, inhibitory substances which mask the effect of stimulatory factors produced in allogeneic mixtures may also be present in the 48-hr to 120-hr supernatants. Thus, of the soluble factors recently described, only EF has been at least partially characterized with respect to physicochemical properties. Consequently, although they act similarly biologically, whether one or more of these mediators are identical awaits further experimentation.

In Table I we have shown that antigenic stimulation affects a relatively small number of cells in the thymus in such a way that they respond specifically to it again on restimulation. This response on the part of primed thymocytes was manifest by the nonspecific enhancement of the response of normal spleen cells to an unrelated antigen. Thus, the addition of TT to mixed cell cultures containing 10 million or 1 million, but not 0.1 million, TT-primed thymocytes resulted in the augmentation of the anti-SRBC hemolytic plaque response. These data support the concept that a portion of T cell memory resides in the immunocompetent cells of the thymus. In addition, tenfold fewer primed
Specific enhancement of immune responses

Thymocytes than primed spleen cells are needed to produce comparable degrees of enhancement. Specific stimulation of mixed cultures containing 1 million TT-primed thymocytes produced augmentation of the PFC response (Table I), whereas similar treatment of cultures containing 1 million primed spleen cells resulted in a slightly depressed plaque response (Table II). Mixed cultures with 10 million primed spleen cells, when stimulated with TT, showed enhancement (Table II). These data suggest that there may be more antigen-reactive cells in the thymus than in the spleen, or that T cells from the thymus, which are capable of responding to anamnestic stimulation in vitro, are more reactive than similar types of memory cells from the spleen. On the other hand, there may be no real difference in the number of EF-producing cells from these two lymphoid organs. In fact, with respect to rosette-forming, antigen-binding cells, tenfold more are found in the spleen than in the thymus (39). It is possible that the disparity in the number of spleen cells or thymocytes needed to produce comparable degrees of enhancement reflects the presence of a more balanced regulatory control mechanism in the spleen in the form of inhibitor-releasing, lymphoid cells which can antagonize the effects of EF-releasing components.

In conclusion, specific antigenic stimulation of thymocytes from primed animals results in the release of a soluble factor which can nonspecifically amplify the humoral immune response of normal spleen cells that are responding to a heterologous, non-cross-reacting antigen. A factor with similar physicochemical properties is also released in vitro by anamnestic stimulation of primed spleen cells. The mediator is most likely protein in nature. An effector molecule such as the one we have described may mediate cellular interactions between T cells and B cells in the induction of antibody synthesis.

Summary

In short-term cultures of thymocytes from tetanus toxoid-immunized mice, the addition of 1 ng of toxoid generated the release of a soluble factor which was capable of enhancing the immune response to a heterologous immunogen. The
addition of supernatants from such cultures to assay cultures of sheep erythrocyte-stimulated normal spleen cells produced a significant augmentation of the hemolytic plaque response. Culture fluid from similar cultures of normal thymocytes or primed thymocytes cultured without the priming antigen were inactive. The enhancing factor was nondialyzable, heat stable (56°C, 30 min), resistant to DNase and RNase, but was inactivated by protease. A factor produced by specifically stimulated primed spleen cells had similar characteristics. In toxoid-stimulated, mixed cell cultures containing primed thymocytes or spleen cells and normal spleen cells, tenfold fewer thymocytes than spleen cells were needed to produce a comparable degree of enhancement of the anti-sheep erythrocyte plaque-forming cell response.

We thank Miss Celia Velletri for excellent technical help.

REFERENCES


