STUDIES OF PPLO INFECTION

V. INHIBITION OF LYMPHOCYTE MITOSIS AND ANTIBODY FORMATION
BY MYCOPLASMAL EXTRACTS*

BY MICHAEL S. SIMBERKOFF, M.D., G. JEANETTE THORBECKE,§ M.D., AND
LEWIS THOMAS, M.D.

(From the Departments of Medicine and Pathology, New York University School of
Medicine, New York 10016)

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Inhibition of the mitotic reaction of lymphocytes to phytohemagglutinin
(PHA) by certain species of mycoplasmas was described by Copperman and
Morton (1). These investigators showed that suspensions of whole myco-
plasmas, as well as aqueous extracts of frozen-thawed organisms (2), prevented
the reaction while in contact with the cells; reactivity was restored when the
lymphocytes were washed.

In view of the ubiquity of mycoplasmas as contaminants of most tissue cul-
ture lines, as well as the possibility that these agents may play roles still un-
recognized in the pathogenesis of disease, it is of importance to determine the
mechanism underlying this phenomenon. Recent reports have suggested that
the mycoplasmas inhibit lymphocyte transformation by competitive metabo-
лизum of arginine (3, 4, 5).

The present report presents additional evidence that the arginine dihydrolase
enzyme system of mycoplasmas is responsible for lymphocyte inhibition. It
will also be shown that the response of lymphocytes to antigenic stimula-
tion is similarly inhibited, and the in vitro production of antibody by lymph node ex-
plants is prevented.

Material and Methods

Preparation of Mycoplasmal Extract.—Mycoplasmas were grown at 37°C in broth media,
as previously described (6), supplemented with 0.5% arginine or 0.5% glucose. Titers of the
organisms at the time of harvest were generally 10⁶ CFU/ml. The mycoplasmas were sedi-
mented by centrifugation and washed three times in 0.85% NaCl. They were then suspended

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§ Recipient of Career Development Award, United States Public Health Service K3-GM-
15, 522.
to a volume \( \frac{1}{2} \)\textsuperscript{th} of the original broth in distilled water and disrupted by 20 cycles of freezing and thawing in a dry ice-acetone bath which resulted in the killing of all organisms. The cell membranes of the disrupted organisms were removed by centrifugation at 30,000 \( g \), and the aqueous supernatant (50 \( \times \)) was diluted in tissue culture medium to 1 \( \times \) prior to addition to cultures, unless otherwise indicated.

**Lymphocytes.**—Cultures of human lymphocytes were prepared from heparinized blood drawn by aseptic venipuncture. The blood was allowed to stand at 37°C for 1–2 hr before the leukocyte-rich plasma was collected. The lymphocytes were separated by passage of the plasma through a glass wool column which restrained most of the other nucleated cells (7). The lymphocytes were washed and suspended in Eagle’s minimal essential medium (MEM), containing 2 mM L-glutamine, 100 units of penicillin, and 100 \( \mu \)g streptomycin/ml with 20% autologous plasma. The concentration of cells was adjusted to 0.5–0.75 \( \times \) 10\( ^{6} \)/ml and the suspension was dispensed in 4 ml aliquots in sterile 16 \( \times \) 125 mm culture tubes.

Mitotic stimulus to the lymphocytes was provided by the addition of PHA, tuberculin, or by the “mixed lymphocyte reaction” (8). Phytohemagglutinin (PHA-M, Difco Laboratories, Detroit, Mich.), 0.1 ml, was added to appropriate tubes. Tuberculin (PPD), Parke, Davis & Co., Detroit, Mich.), 5 \( \mu \)g, was added to lymphocytes of a tuberculin-sensitive donor. The mixed lymphocyte reaction was carried out by combining 2 ml cell suspensions for two donors. Mycoplasmal extract, 0.1 ml, was then added, and the tubes were gassed with 5% \( \text{CO}_2 \)-air incubated at 37°C.

Lymphocytic response to these stimuli was determined by incorporation of \( ^{14} \)C-labeled thymidine (9) and by morphological criteria. 24 hr prior to harvesting, 0.1 \( \mu \)c of \( ^{14} \)C-thymidine (specific activity 54.1 mc/mmole) was added to triplicate tubes. At the end of the incubation period, the cells were sedimented, washed once with 0.85% NaCl, twice with cold trichloroacetic acid, and twice with cold methanol. The material was then dried at room temperature overnight, dissolved in Hyamine (Packard Instrument Co., Downers Grove, Ill.), and transferred to vials containing Liquidfluor (Packard) in toluene. Radioactivity was counted in a Packard Tri-Carb scintillation counter.

Cells from duplicate tubes without labeled thymidine were harvested at the same time, spread on coverslips, and stained with acetic orcein. Cell viability was determined by exclusion of trypan blue.

**Antibody Formation in Vitro.**—Adult male New Zealand white rabbits were immunized with a single dose of 2 \( \times \) 200 Lf diphtheria toxoid (DT, Lederle Laboratories, Pearl River, N. Y.), injected into the hind footpads. Popliteal lymph nodes from these animals were removed for tissue culture 2 months or longer after immunization.

The in vitro production of antibody was investigated as previously described (10, 11). Approximately 20 mg of 1–2 mm lymph node fragments was distributed over the walls of 16 \( \times \) 125 mm screw-capped roller tubes. Each tube received 1 ml of MEM containing 25% normal rabbit serum. Within each experiment, duplicate tubes received similar additions of antigen (1 Lf DT) and mycoplasma (0.05 ml of 1 \( \times \) or 50 \( \times \) concentrated extract). Antigen was left with the tissue for the initial 7–8 hr of culture, and then rinsed off with Hanks’ basic saline solution (BSS). The medium was changed every 3 days and the cultures were maintained from 12–18 days. In some experiments, the tissue was exposed to \( ^{14} \)C-amino acid as described below.

**Protein Production by Other Cell Types.**—The parenchymal type hepatoma cell line H411-Ec3 (12) was grown on the wall of 15 ml plastic culture flasks in Dulbecco’s medium. Cells from a line derived from a human lymph node, SK-LN-1 (13), were grown in modified McCoy’s medium (Grand Island Biological Co., Grand Island, N. Y.) containing 15% fetal calf serum. Mycoplasmal extract (0.2 ml per 5 ml medium) was added and left for 24 or 48 hr with these cells before their exposure to medium containing \( ^{14} \)C-amino acids.

In order to study serum protein synthesis, the cells were incubated at 37°C for 24 hr in
modified MEM containing $^{13}$C-labeled lysine (1 $\mu$C/ml) and isoleucine (1 $\mu$C/ml) of specific activity 100–200 mc/mmole. The culture fluids were dialyzed, lyophilized, and redissolved for characterization by immunoelectrophoresis (IE) and radioautography as described elsewhere (14).

This method can also be used for the demonstration of antibody synthesis and differentiates between release of intracellularly stored antibody and synthesis (14). The "carrier-system" in the IE patterns consisted in such cases of an antigen-antibody precipitation arc made by precipitation of strong anti-DT, after subjection to electrophoresis, by dilute DT.

Antibody Formation In Vivo.—Mycoplasma extract was injected into adult Sprague-Dawley rats and Swiss albino mice according to a variety of schedules. Sera were obtained from these animals 5–7 days after the intravenous injection of sheep red blood cells. Hemagglutinin titers of the sera, inactivated at 56°C, were measured by microtiter.

RESULTS

The Effect of Various Mycoplasmas on Lymphocyte Reactivity.—The effect of 10 species of mycoplasma on the response of lymphocytes is shown in Table I. Suspensions of mycoplasmas, killed by freeze-thawing, were added to lymphocyte cultures stimulated with PHA. Five of these completely inhibited incorporation of labeled nucleotide into the lymphocytes. Comparable extracts of the remaining five had no effect.

Arginine is known to serve as an important source of energy for certain species of mycoplasma through the arginine dihydrolase enzyme system (15). Five of the strains studied utilize arginine in this way; five do not. It was found that the capacity to inhibit lymphocyte responsiveness was correlated in each instance with the requirement for supplemental arginine, as is indicated in Table I. In each case, inhibition of nucleotide uptake was accompanied by complete suppression of mitosis and transformation to blast cells, estimated by direct examination of Giemsa-stained films.

The viability of lymphocytes was not affected by any of the mycoplasmas tested as judged by morphological study of stained smears and by the exclusion of trypan blue in fresh preparations.

Properties of the Inhibitory Extract.—Extracts from Mycoplasma arthritidis were prepared and treated in a variety of ways in an attempt to purify the lymphocyte inhibiting factor. The results are shown in Table II. The supernatant fraction of freeze-thaw disrupted organisms centrifuged at 30,000 $g$ or 150,000 $g$ inhibited lymphocytes, indicating that the factor was not associated with the antigentic membrane of the mycoplasma (16) nor with its ribosomal fraction (17). Dialysis of the 30,000 $g$ supernatant against saline and extraction three times with cold ether did not diminish its inhibitory capacity. When the supernatant was heated at 56°C for 2 hr, however, it was no longer inhibitory. Trypsin did not affect the extract, but treatment with cysteine-activated papain at 37°C for 2 hr destroyed all activity. Nonactivated papain had no effect on the system. The results suggest that the active portion of the mycoplasma extract is a nondialyzable, heat-labile protein.
### TABLE I

**Effect of Various Species of Mycoplasma on the Lymphocyte Response to PHA**

| Species          | Arginine metabolism | \(^{14}C\)-thymidine incorporated(*) | Inhibition of mitosis and transformation
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm</td>
<td>cpm</td>
</tr>
</tbody>
</table>
| *M. arthritidis* | Yes                 | 16,622                             | 25                               | ++++
| *M. hominis I*   | Yes                 | 16,622                             | 75                               | ++++
| *M. orale I*     | Yes                 | 11,649                             | 11                               | ++++
| *M. orale II*    | Yes                 | 11,497                             | 35                               | ++++
| *M. salivarium*  | Yes                 | 11,649                             | 465                              | ++++
| *M. pneumoniae*  | No                  | 16,573                             | 14,140                           | 0
| *M. gallisepticum* | No               | 16,622                             | 14,846                           | 0
| *M. pulmonis*    | No                  | 16,573                             | 13,980                           | 0
| *M. neurolyticum* | No                | 16,622                             | 10,757                           | 0
| *M. fermentans*  | No                  | 8,771                              | 7,281                            | 0

* By lymphocytes during the final 24 hr of a 72 hr incubation period. The results are the average of triplicate samples.

† Control human lymphocyte cultures contain approximately 2 million cells in 4 ml medium (MEM with 20% autologous plasma) and 0.1 ml PHA-M.

§ 0.1 ml of the freeze-thaw disrupted organisms added to cultures otherwise identical to control.

|| Inhibition of morphological transformation of cells compared to controls. ++++ denotes complete inhibition, while 0 denotes no inhibition.

### TABLE II

**Effect of Extracts from *M. arthritidis*, Prepared in Various Ways, on Lymphocyte Response to PHA**

<table>
<thead>
<tr>
<th>Extract treatment</th>
<th>(^{14}C)-thymidine incorporated(*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
</tr>
<tr>
<td><em>M. arthritidis</em>, freeze-thawed</td>
<td></td>
</tr>
<tr>
<td>Supernatant, 30,000 g</td>
<td>16,622</td>
</tr>
<tr>
<td>&quot; 150,000 g</td>
<td>16,238</td>
</tr>
<tr>
<td>&quot; 30,000 g, dialyzed</td>
<td>20,582</td>
</tr>
<tr>
<td>&quot; heated 56°C, 2 hr</td>
<td>16,238</td>
</tr>
<tr>
<td>&quot; either extracted</td>
<td></td>
</tr>
<tr>
<td>&quot; papain treated¶</td>
<td>33,725</td>
</tr>
<tr>
<td></td>
<td>14,516</td>
</tr>
</tbody>
</table>

* Mitotic activity of lymphocytes during the final 24 hr of a 72 hr incubation period.

† Control human lymphocyte cultures contain approximately 2 million cells in 4 ml medium and 0.1 ml PHA-M.

§ 0.1 ml of organism or extract treated as indicated added to cultures otherwise identical with control.

|| Aqueous material extracted three times with equal volumes of cold diethyl ether.

¶ Mycoplasmal extract was treated with 0.5 mg/ml cysteine activated crystallized papain, 37°C for 2 hr. The papain was then inactivated by dialysis of the solution.
Reversibility of the Effect of Mycoplasmal Extract.—The response of lymphocytes to PHA is the result of binding between PHA and receptors on the cell surface (18). The mycoplasmal extract did not interfere with this process. Cells exposed to PHA and extract, and then washed, subsequently underwent blast transformation without additional PHA (Table III).

Removal of the extract by washing permitted the lymphocytes to regain responsiveness to PHA, although their capacity to incorporate thymidine was somewhat less than that of untreated controls. As is also shown in Table III, cells treated in the first 24 hr with extract were less reactive to PHA added after this time. The spontaneous activity of cells, as indicated by the incorporation of nucleotide in the absence of PHA, was also suppressed by the extract.

### Table III

<table>
<thead>
<tr>
<th>Additives to initial medium</th>
<th>Additives to medium after change at 24 hr†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>None</td>
<td>182</td>
</tr>
<tr>
<td>PHA§</td>
<td>32,305</td>
</tr>
<tr>
<td>Extract‖</td>
<td>91</td>
</tr>
<tr>
<td>PHA + Extract</td>
<td>21,942</td>
</tr>
</tbody>
</table>

*Mitotic activity of lymphocytes expressed as cpm ¹⁴C-thymidine incorporated during the final 24 hr of a 96 hr incubation period.
†24 hr after initiation of the culture, medium was removed, the cells were washed and resuspended in fresh medium with additives as indicated.
§PHA-M, 0.1 ml added to each tube.
‖Extract, 0.1 ml of the 30,000 g supernatant of freeze-thaw disrupted mycoplasma added to each tube.

Although the addition of mycoplasmal extract to lymphocyte cultures 24 hr after stimulation seemed to block subsequent mitotic activity, it was of interest to determine whether this effect would be noted after the appearance of morphologically transformed cells in the cultures (19). Fig. 1 shows the results of an experiment in which the extract was added to lymphocyte cultures 3 days after stimulation with PHA. Although nucleotide incorporation dropped to about 25% of the control value, it nonetheless continued at a significant rate, and, within another 3 days, was again increasing.

Effect of Arginine on Mycoplasmal Extract.—The observation that inhibition by the factor was correlated with the property of arginine utilization by certain mycoplasma species suggested that the inhibitor might be an enzyme involved in the metabolism of this amino acid. To explore this possibility, the effect of supplemental arginine on lymphocytes treated with PHA extract was investigated. The results are shown in Table IV.
MEM contains 10.5 mg% arginine and, in addition, this amino acid is a constituent of plasma. The addition of excess arginine, as seen in Table IV, caused reversal of the inhibitory effect of mycoplasmal extract. The amounts of arginine required to reverse inhibition varied with the concentration of extract em-

![Graph showing counts per minute](image)

**Fig. 1.** Mitotic activity of lymphocytes expressed as cpm ¹⁴C-thymidine incorporated during the 24 hr period prior to harvesting of the cells on the day indicated. PHA-M, 0.1 ml was added to the tubes indicated at the initiation of the culture, while *M. arthritidis* extract, 0.1 ml of the I X solution, was added to the tubes indicated at the start or on day 3 of incubation.

**TABLE IV**

<table>
<thead>
<tr>
<th>Supplements to Medium</th>
<th>None</th>
<th>Arginine 20 mg</th>
<th>Arginine 10 mg</th>
<th>Arginine 5 mg</th>
<th>Arginine 20 mg</th>
<th>Citrulline 20 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13,399</td>
<td>12,095</td>
<td>—§</td>
<td>—</td>
<td>8,927</td>
<td></td>
</tr>
<tr>
<td>0.1 ml, 1 X concentrated</td>
<td>9 1,582</td>
<td>162</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>0.1 ml, 3/5 X concentrated</td>
<td>3 11,159</td>
<td>15,436</td>
<td>4,384</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>0.1 ml, 3/10 X concentrated</td>
<td>247 10,029</td>
<td>16,639</td>
<td>15,280</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mitotic activity expressed as cpm ¹⁴C-thymidine incorporated into lymphocytes during final 24 hr of 72 hr incubation period. PHA-M, 0.1 ml was added to each tube.

† Extract, arginine, and citrulline are given as total amount added to 4 ml of cell suspension.

§ Not tested.
ployed. Citrulline is the first product of the arginine dihydrolase system (15). When this amino acid was tested in a similar range of concentrations, no effect was noted.

In order to learn whether the extract acted primarily on the medium or the lymphocytes themselves, experiments were performed with medium which had been pretreated with \textit{M. arthritidis} extract and then heated to destroy enzyme activity. As is shown in Table V, lymphocytes were unable to respond to PHA when suspended in such medium, but the addition of arginine caused complete restoration of the response. At the concentration used, arginine had no influence on the inhibition produced by active extract.

\begin{table}
\centering
\caption{Effect of Preincubation of Medium with \textit{M. arthritidis} Extract on Lymphocyte Response\textsuperscript{*} to PHA}
\begin{tabular}{|l|c|c|}
\hline
Treatment of medium\textsuperscript{†} & Added with lymphocytes & Arginine, 1 mg \\
& to medium & No arginine§ & \\
\hline
Preincubated alone & PHA + active extract & 11 & 13 \\
Preincubated with active extract, then 56°C, 2 hr & PHA & 36 & 9,302 \\
Preincubated with heat-inactivated\textsuperscript{¶} extract & PHA & 11,649 & 9,698 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{*} Mitotic activity expressed as cpm $^{14}$C-thymidine incorporated by lymphocytes during final 24 hr of 72 hr incubation period.

\textsuperscript{†} Media were preincubated at 37°C for 24 hr with or without \textit{M. arthritidis} extract as indicated and then heated at 56°C for 2 hr prior to addition of lymphocytes.

\textsuperscript{§} Arginine, total amount added to 4 ml cell suspension.

\textsuperscript{¶} Extract, 0.1 ml, 30,000 g supernatant added per 4 ml medium as indicated. PHA-M, 0.1 ml added per tube with lymphocytes.

\textit{M. arthritidis}, 1 $\times$ concentrated, contained 0.133 IU of arginine deiminase (assayed by M. I. Rothman and Dr. J. D. Broome). Further evidence that the inhibitory effect on lymphocytes is due to the action of this enzyme was obtained in experiments with an arginine deiminase derived from \textit{Streptococcus faecalis} (20). In concentrations of 0.016 IU/tube or higher, this enzyme caused complete inhibition of the response of human lymphocytes to PHA.

\textit{Antigenicity of the Mycoplasmal Extract}.—Sera from rabbits immunized with various mycoplasmas, as previously described (21), were tested for their effect on the extract in lymphocyte cultures. In these experiments, 0.1 ml amounts of extract and 0.1 ml of 1:5 dilutions of the sera were added to lymphocyte cultures stimulated with PHA. Sera from two rabbits immunized with \textit{M. arthri-
tidis reversed the effect of extracts of that organism on the lymphocytes. These sera also blocked the effects of extracts prepared from *M. hominis I, M. orale I, M. orale II,* and *M. salivarium.* Sera from two rabbits immunized with *M. gallisepticum,* an organism which does not metabolize arginine, did not reverse inhibition. It is of interest that none of these sera blocked the effects of the streptococcal enzyme on the stimulated lymphocytes.

**TABLE VI**

_Effect of L-Canavanine on Lymphocyte Response* to PHA and Reversal by Arginine_

<table>
<thead>
<tr>
<th>L-Canavanine added†</th>
<th>Arginine added‡</th>
<th>5 mg</th>
<th>1 mg</th>
<th>0.5 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>3,665</td>
<td>2,227</td>
<td>3,903</td>
</tr>
<tr>
<td>5 mg</td>
<td>None</td>
<td>30</td>
<td>1,000</td>
<td>899</td>
</tr>
<tr>
<td>2.5 mg</td>
<td></td>
<td>424</td>
<td>2,731</td>
<td>2,333</td>
</tr>
<tr>
<td>1.25 mg</td>
<td></td>
<td>1,902</td>
<td>3,111</td>
<td>3,017</td>
</tr>
</tbody>
</table>

*Mitotic activity expressed as cpm 14C-thymidine incorporated during the last 24 hr of a 72 hr incubation period. Control and test lymphocyte cultures contained 0.1 ml of PHA-M.†Total amount added to 4 ml of lymphocyte culture medium.

**TABLE VII**

_Effect of M. arthritidis Extract on Antigenic Stimulation of Lymphocytes_

<table>
<thead>
<tr>
<th>Incubated lymphocytes</th>
<th>14C-thymidine incorporated*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No extract added</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
</tr>
<tr>
<td>PPD-sensitive cells without PPD</td>
<td>60</td>
</tr>
<tr>
<td>PPD-sensitive cells with PPD, 5-μg</td>
<td>4,067</td>
</tr>
<tr>
<td>Cells of K. S. and E. B. separately</td>
<td>203</td>
</tr>
<tr>
<td>Cells of K. S. and E. B.§ mixed</td>
<td>2,316</td>
</tr>
</tbody>
</table>

*14C-thymidine incorporated during the final 24 hr of 120 hr incubation period. Cultures contained 2 million cells in 4 ml medium.
‡Extract, 0.1 ml, 30,000 g supernatant freeze-thaw disrupted mycoplasma.
§1 million cells from each donor, K. S. and E. B., added to final 4 ml incubation mixture.

_The Effect of L-Canavanine on Lymphocyte Reaction._—L-canavanine, an analogue of arginine, has been shown to inhibit competitively the growth and metabolism of bacteria (22) and a number of human cell cultures in vitro (23). It was therefore of interest to determine its effect on PHA-stimulated lymphocytes. The results are shown in Table VI. 5 mg of canavanine completely blocked thymidine incorporation by lymphocytes while smaller amounts caused
partial inhibition. Supplementation of the medium with arginine resulted in a competitive reversal of the inhibition.

The Effect of Mycoplasmal Extract on Immunologically Stimulated Lymphocytes.—The in vitro response of lymphocytes from immunized donors to contact with specific antigens resembles the reactions of these cells to PHA (24); the cells undergo mitotic transformation to blast forms and the uptake of nucleotides is correspondingly stimulated. In order to learn whether this reaction is inhibited by the mycoplasmal extract, the following experiments were performed.

### TABLE VIII

**Effect of M. arthritidis Extract on the Secondary Immune Response to Diptheria Toxoid by Rabbit Lymph Nodes in Vitro**

<table>
<thead>
<tr>
<th>Exposure to extract*</th>
<th>Day of antigen addition</th>
<th>Reciprocal of peak titer†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>Exp. I</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>16,000</td>
</tr>
<tr>
<td>Throughout, 1 × concentrated</td>
<td>0</td>
<td>1,000</td>
</tr>
<tr>
<td>Throughout, 50 × concentrated</td>
<td>0</td>
<td>&lt;2</td>
</tr>
<tr>
<td>First 24 hr “ “ “</td>
<td>0</td>
<td>24,000</td>
</tr>
<tr>
<td>First 3 days “ “ “</td>
<td>0</td>
<td>2,000</td>
</tr>
<tr>
<td>First 6 days “ “ “</td>
<td>0</td>
<td>4,000</td>
</tr>
<tr>
<td>First 9 days “ “ “</td>
<td>0</td>
<td>4,000</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>4,000</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>48</td>
</tr>
<tr>
<td>First 6 days “ “ “</td>
<td>6</td>
<td>1,500</td>
</tr>
</tbody>
</table>

*0.5 ml of varying concentrations of 30,000 g supernatant, freeze-thaw disrupted organisms.
† Passive hemagglutinin titer of third medium change after addition of 1 Lf DT left for 7 hr with lymph node fragments at initiation of cultures. Medium was changed every 3 days.

Lymphocytes from tuberculin-sensitive donors were cultured in medium containing tuberculin (PPD) and *M. arthritidis* extract was added. The results are shown in Table VII.

Lymphocytes from unrelated donors were combined in approximately equal numbers and cultured; the mitotic response of such mixed cell populations has been shown to be a reflection of homograft incompatibility (25). The effect of *M. arthritidis* extract on this reaction is also shown in Table VII.

Both the in vitro reaction to tuberculin and the mixed lymphocyte reaction were completely inhibited by the addition of this extract.

The Effect of Mycoplasmal Extract on the Secondary Immune Response in Vitro.—Lymph node explants from rabbits previously immunized against diphtheria toxoid were found to produce antibody in vitro within 6 days after exposure to
antigen in titers ranging from 1:2,000 to 1:32,000. Peak titers were usually encountered on the 9th day of culture, and antibody continued through at least the 15th day.

*M. arthritidis* extract completely prevented the production of antibody when present throughout the period of culture. The results of an illustrative experiment are shown in Table VIII. It was noted that explants cultured with mycoplasma extract showed little or no evidence of proliferation at the periphery when compared with controls. Extracts of *M. hominis I* had the same inhibitory action on antibody formation and cell proliferation, while an extract of *M. gallisepticum* (which had been shown to be without effect on the PHA response) caused no inhibition.

Removal of the mycoplasmal extract, by changing the medium in which the explants were cultured, resulted in prompt antibody production, indicating that the extract did not interfere with the initial reaction between antigen and...
FIG. 3. Radioautograms (RA) of immunoelectrophoretic (IE) patterns (bottom) prepared with culture fluids from lymph node fragments. The tissues were grown for 13 days after induction of a secondary response to diphtheria toxoid (DT) in minimal Eagle's medium with 20% normal rabbit serum. To some tubes, 0.05 ml of 50 X concentrated *M. arthritidis* extract (MYC) per ml was added, either for the first 6 days only, or for the full 13 days. The 14C-amino acid-containing medium was then left with the tissue for 24 hr. The carrier patterns were developed from rabbit antiserum to DT (RAT) with a sheep antiserum to rabbit serum (anti-rabbit) or with DT. Note strong labeling of γ-globulin (IgG) in all cultures except the one left with *M. arthritidis* extract for the full 13 days, and labeling of antibody after exposure to DT only when the tissues were left for 7 or 13 days in the absence of the *M. arthritidis* extract.
Fig. 4. Radioautograms (RA) of immunoelectrophoretic (IE) patterns (bottom) prepared with culture fluids from rat hepatoma cell line, H411-Ec3. The cells were grown in Dulbecco's medium in the presence of 0.05 ml of a 50 × concentrated M. arthritidis extract (MYC) per ml of medium for 48 hr prior to addition of the ^14C^-amino acid-containing medium with a similar M. arthritidis extract concentration for an additional 24 hr. Carrier patterns were developed from undiluted or 1:4 diluted rat serum (RS) by two different rabbit antisera to whole rat serum (anti-rat). Note identical labeling of a variety of serum proteins in cultures regardless of exposure to M. arthritidis extract.
cell receptors. Explants held in a state of inhibition for periods as long as 9 days remained capable of producing antibody once the extract was removed.

Addition of 1 mg of canavanine per milliliter at each medium change also markedly diminished antibody production in these cultures. Removal of the canavanine, however, did not result in recovery of the antibody formation.

![Fig. 5. Radioautograms (RA) of immunoelectrophoretic (IE) patterns (bottom) prepared with culture fluids from human lymphoid cell line, SK-LN-1. The cells were grown in McCoy's medium in the presence of 0.05 ml of a 50 × concentrated *M. arthritidis* (MYC) extract per ml of medium for 24 hr and were then left with the 14C-amino acid-containing medium with a similar *M. arthritidis* extract concentration for an additional 24 hr. Carrier patterns were developed from whole human serum (HuS) with rabbit antiserum to whole human serum (anti-human). Note slight reduction in labeling of IgG in cultures 3 and 4, containing *M. arthritidis* extract, as compared to control cultures 1 and 2. Cultures 2 and 4 contained three times the number of cells contained in cultures 1 and 3 during the period of exposure to 14C-amino acid.

It will be noted in Table VIII that control explants, not exposed to antigen, produced low levels of antibody throughout the period of incubation, and that this spontaneous activity was also inhibited by mycoplasmal extract. When tissues were treated with extract for 6 days, and then washed and exposed to antigen, their capacity to produce antibody was significantly greater than that of control cells.

Although mycoplasmal extract prevented the initiation of antibody production, it was not capable of shutting off production when it had already begun.
This point is illustrated in Fig. 2. When explants were exposed to antigen and then cultured for 6 days before the addition of extract, antibody continued to be formed during the following 9 days. The level of antibody did not increase after the 6th day, as did the control, and the rate of decline after the 9th day was greater, but it was clear that the explants continued to produce antibody despite the presence of mycoplasmal extract. The results were in sharp contrast to the total inhibition seen when the extract was introduced simultaneously with the antigen.

The Effect of Mycoplasmal Extract on Protein Production in Vitro

Although radioautography and immunoelectrophoresis patterns do not provide a quantitative measure of serum protein production, previous studies have shown it can be used for semiquantitative comparisons between levels of serum protein synthesis in different tissues (14). Incorporation of 14C–amino acids into γ-globulin normally occurs in the lymph node fragments used for these studies throughout the period of observation. Although exposure to antigen enhances this production somewhat in the sensitized tissue, γ-globulin formation also occurs in the absence of antigenic exposure. Fig. 3 illustrates the production of IgG and antibody in lymph node fragments during the 13th day after initiation of the culture. It can be seen that the presence of mycoplasmal extract for 6 days from the outset of the culture period did not affect the final levels of IgG or antibody production, while the continuous presence of the extract not only eliminated antibody synthesis, but also greatly reduced the IgG synthesis.

Production of serum proteins, including albumin, transferrin, and various α and β-globulins by the rat hepatoma line, H411-Ec3 (26), was not significantly affected by the extract. Even after growing for 48 hr in the presence of extract concentrations which completely inhibited induction of a secondary response in lymph node fragments, these parenchymal type liver cells actively incorporated 14C–amino acids into serum proteins (Fig. 4). The growth rate of these cells was also unaffected by the mycoplasmal extract over a period of 48 hr.

The human lymphoid cells, SK-LN-1, grown as a continuous suspension culture, continues to form immune globulins, although at a slightly reduced level, during and after exposure to M. arthritidis extract (Fig. 5).

The Effect of Mycoplasmal Extract on Antibody Production in Vivo

In view of the capacity of mycoplasmal extracts to suppress antibody production in vitro, experiments were designed to determine whether comparable effects could be obtained in live animals. Rats and mice were given daily injections, by intravenous or intraperitoneal routes, of 0.1–0.5 ml of the 50 × M. arthritidis extract. A suspension of sheep erythrocytes was injected by vein after the first injection of extract, and the levels of hemagglutinating antibody were determined on the 7th day. No inhibition of antibody formation was demonstrable under these conditions.

The Effect of L-Asparaginase on Lymphocyte Reactions

A possibility raised
by these findings was that the arginase enzyme uniquely affects lymphocytic responsiveness. The effects of L-asparaginase on these cells was therefore studied. The results with two enzyme preparations, Escherichia coli and agouti serum are shown in Table IX. While the E. coli enzyme completely inhibited the incorporation of thymidine into PHA-stimulated lymphocytes and antibody production in vitro, comparable amounts of agouti serum were ineffective. It is known that while the agouti serum asparaginase is highly specific, the E. coli enzyme is also capable of degrading glutamine (27). It is possible that this accounts for the difference between the two preparations. In fact, addition of both

TABLE IX

**Effect of "Asparaginase" on Lymphocyte Response to PHA and Secondary Antibody Response in Vitro**

<table>
<thead>
<tr>
<th>Added test material§</th>
<th>Control</th>
<th>+ Test material</th>
<th>Inhibition secondary antibody response¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparaginase, 1 IU¶</td>
<td>9,682</td>
<td>566</td>
<td>++</td>
</tr>
<tr>
<td>Asparaginase, 5 IU</td>
<td>15,105</td>
<td>12</td>
<td>++</td>
</tr>
<tr>
<td>Asparaginase, 10 IU</td>
<td>9,682</td>
<td>7</td>
<td>+++</td>
</tr>
<tr>
<td>Agouti serum, 2.5 IU</td>
<td>5,112</td>
<td>1,242</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mitotic activity expressed as cpm 14C-thymidine incorporated during the last 24 hr of a 72 hr incubation period. Control and test lymphocyte culture tubes contained 0.1 ml PHA-M.
† Anti-diphtheria toxoid response by sensitized rabbit lymph node fragments after exposure to DT in vitro.
§ Total amounts added to 4 ml culture medium for human lymphocytes.
¶ Results expressed as comparison to control cultures. +++ denotes virtually complete suppression; 0, negligible suppression of antibody formation. The E. coli asparaginase (1 or 10 IU/ml) or agouti serum (1 IU/ml medium) was added with each medium change.
¶§ Asparaginase (Sigma Chemical Co., St. Louis, Mo). purified from E. coli 30 IU/mg.

L-asparagine and L-glutamine to the medium along with the E. coli enzyme did not reverse its effect. As with the mycoplasmal extract, complete reversibility of inhibition by washing and medium change was demonstrated. Supplementation of the medium with arginine, however, did not reverse its effect. Thus, this enzyme, which does not affect arginine metabolism, clearly affects lymphocytic reactivity, although the mechanism of its action remains to be determined.

**DISCUSSION**

The present study indicates that extracts of certain mycoplasma inhibit the proliferative response of lymphocytes to a variety of stimuli. In addition to preventing PHA-induced blast transformation, as described earlier by Copperman and Morton (2), these extracts also block the response of sensitized lymphocytes to tuberculin and homograft antigens, and the secondary production of
antibody to diphtheria toxoid in vitro. The mechanism of all these effects is probably through inhibition of blast cell transformation and, consequently, of proliferation. Agents which inhibit cell proliferation, such as BUDR (5-bromo-deoxyuridine), are known to inhibit the secondary immune response in vitro when applied during the first few days after exposure of tissue to antigen (28).

Mycoplasma extract must be present throughout the cell incubation period to prevent lymphocytic transformation or the secondary antibody response. If the extract is removed after 24 hr, thymidine incorporation in lymphocytes stimulated with PHA is almost completely restored. The fact that restoration was usually partial could be due to slight toxicity of the extract for lymphocytes, although viable cell counts with trypan blue gave no evidence for toxicity. A better explanation may be that the cells treated with extract and PHA for the first 24 hr are simply deprived of this period of PHA-stimulated activity, and have, therefore, a shorter total time for the cumulative response to PHA.

No evidence of any toxicity of the mycoplasma extract on lymph node fragments has been obtained from the studies on antibody formation, even though concentration of the extract used was considerably higher than in peripheral lymphocyte experiments. On the contrary, preservation of immune responsiveness was noted in lymph node cultures incubated in the presence of mycoplasmal extract during a 6 day period prior to addition of the antigen. Comparable cultures held for this period without extract are capable of only a very low antibody response upon addition of antigen on the 6th day. The extract may have preserved the reactivity of the tissue by preventing spontaneous blast formation and dedifferentiation of the "memory" cells in the lymph node fragments.

A number of findings support the view that the active portion of the mycoplasmal extract is a protein. It is present in the supernatant fraction of 150,000 g centrifugation. Activity is preserved after ether extraction and dialysis, but is lost after heating at 56°C and after treatment with activated papain. A previous report indicated that the mycoplasmal extract was not affected by trypsin (3). The apparent difference in sensitivity to two proteolytic enzymes may be due to presence of trypsin inhibitors in the crude extract.

The following data suggest that the lymphocyte inhibitor is arginine deiminase, an enzyme known to account for as much as 10% of the protein content of M. arthritidis (15). All of the mycoplasma species that inhibit the response of lymphocytes to PHA require supplemental arginine for growth. The addition of adequate amounts of arginine to medium containing extract restores the normal responsiveness of the lymphocytes to PHA. Moreover, pretreatment of the medium with extract, followed by heat inactivation of the latter, yields medium in which the PHA reaction does not occur, but supplementation of this medium with arginine restores the reaction. Finally, an arginase derived from S. faecalis caused similar inhibition of the PHA response of lymphocytes and also inhibited antibody formation by lymph node explants. It is of interest that
antiserum against *M. arthritidis* caused inhibition of the extracts from all of the arginine requiring mycoplasmas studied, indicating that the enzymes from all are closely related or identical, but did not neutralize the streptococcal enzyme.

The capacity of canavanine to inhibit both the lymphocyte response to PHA and the secondary immune response of lymph node fragments in vitro is consistent with the observed effect of mycoplasmal extracts. Canavanine is effective as a competitive analogue of arginine in other circumstances (21), and would be expected to mimic arginine depletion in this system, except that its effect should be, and is, less reversible.

Continued synthesis of antibody at somewhat reduced levels is noted in cultures which were treated for 6 days with mycoplasmal extracts after antigenic stimulation and then washed to remove the extract. Similarly, continuous immune globulin synthesis by a human lymphoid cell line is somewhat reduced, but not abolished. Rat hepatoma cells, on the contrary, continue unaltered levels of serum protein production in the presence of the mycoplasmal enzyme. These findings suggest that the memory cell for the immune response, the small lymphocyte, is dependent on exogenous arginine for proliferation. Its progeny, however, like hepatoma cells, can continue to function in the absence of this amino acid. This may be due to accumulated stores, decreased requirement for arginine, or to formation of enzymes to synthesize the amino acid endogenously. The enzyme content of transformed cells is quantitatively and perhaps qualitatively increased in comparison to the small lymphocyte (29).

The experiments with L-asparaginase indicate that the action of arginase on lymphocytes cannot be regarded as unique. The mode of action of the *E. coli* enzyme remains in doubt since neither L-asparagine, nor L-glutamine, nor L-arginine were capable of reversing its effect. However, it is clear that arginine depletion is not involved in its inhibitory effect, and that other enzymes may have a similar effect.

The possibility exists that the observed effects of mycoplasmas on lymphocytes may play a role in the pathogenesis of disease caused by these agents, although no evidence bearing on the point was obtained in these studies. The failure to inhibit antibody formation in rats and mice given repeated injections of extract is most likely due to failure to bring about sustained arginine depletion for long enough periods in vivo. It may be that the situation in a localized infection with living mycoplasmas in close proximity to the surface of lymphocytes, might well result in interference with immune responsiveness. Further studies on the effects of infection with live mycoplasmas are in progress.

**SUMMARY**

Extracts of five arginine-utilizing mycoplasmas inhibit PHA-induced lymphocyte mitosis, while extracts of five glucose-utilizing mycoplasmas do not. Evidence is presented supporting the view that the inhibitory factor is the enzyme arginine deiminase. This enzyme inhibits the reactions of human
lymphocytes to antigens as well as PHA, and the secondary production of antibody by rabbit lymph node fragments in vitro. Addition of enzyme to the cells several days after the initial mitotic or antigenic stimulus reduces, but does not abolish, further cellular activity. The production of serum proteins by hepatoma cells is totally unaffected by the mycoplasmal extract. It is concluded that arginine is an essential amino acid for the small lymphocyte, but not for the transformed cell nor for a number of other cell types. Suggestive evidence has been obtained that other enzymes similarly affect lymphocyte reactions.

The authors would like to thank Mr. I. Rothman and Dr. J. D. Broome for supplying *Streptococcus faecalis* arginine deiminase and for assaying that enzyme in the mycoplasmal extract, Dr. J. D. Broome for supplying the agouti serum, and Dr. B. Clarkson for supplying the SK-LN-1 cells.

**BIBLIOGRAPHY**


