Immunologic Memory Of Phytohemagglutinin-Stimulated Lymphocytes

By Jerome I. Brody and Henry D. Soltys

THE PURPOSE of this study was to delineate the durability and persistence of immunologic recall in the normal peripheral blood lymphocyte using methods which might reflect the importance of the manner by which immunologic information is acquired. This was done in the present investigation with the technic of bacterial adherence, in which circumferential adhesion of a microorganism to the lymphocyte surface indicates antibody production by this cell. The assays described below compared the immune reactivity of phytohemagglutinin-stimulated lymphocytes against E. coli, a natural, lifelong immunogen, and against Salmonella, one which is acquired and deliberately given when a special need arises.

Although phytohemagglutinin (PHA) is considered a general biosynthetic and metabolic stimulant of lymphocytes, it is also able, under appropriate circumstances, to exercise its biologic activity on a more specific, genetic level. This occurs, for example, when it predictably alters the proportions of genetically controlled forms of lymphocyte lactate and malate dehydrogenases. Theoretically, on an analogous basis, it could potentially influence lymphocyte immune responses toward various antigens depending on their type, the means by which cellular contact was initiated, and on the immunologic memory ultimately generated and stored within this cell.

Methods and Materials

Lymphocyte Donors

Peripheral blood lymphocytes were obtained from 7 healthy members of the hospital professional staff. Of these, 2 had never been immunized against Salmonella. The remainder had been given commercial polyvalent Salmonella vaccine 1 month to 8 years prior to this study.*

Cell Culture Procedures

To harvest lymphocytes, venous blood was defibrinated and the cells separated by dextran sedimentation and differential centrifugation as previously described in detail. From the Department of Medicine, the Graduate Hospital of the University of Pennsylvania, and the University of Pennsylvania School of Medicine, Philadelphia, Pa. First submitted April 18, 1969; accepted for publication July 3, 1969. Supported by USPHS training grant 5 TI CA 5159, USPHS research grant CA 07000 and an institutional grant from the American Cancer Society.

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*Lymphocyte donors 1, 2, 3, and 7 (Table 1) received vaccine in this laboratory made by Lederle Division, American Cyanamid Company, Pearl River, N.Y.
For each experiment quadruplicate cultures were made in Opti-clear, disposable vials, each holding 10 million lymphocytes, 3 ml. autologous, heparinized plasma, and 5 ml. TC 190. The first vial received no additive. The second culture was given .05 ml. of a PHA-M stock solution prepared by dissolving 1 bottle of PHA-M, as received from the supplier, in 5 ml. buffer. To directly test donor lymphocyte responses to the different bacteria, 0.5 ml. of a standardized, soluble E.coli and Salmonella antigen were placed in cultures three and four respectively. Tissue culture reactivity of lymphocytes from donors 1, 2, 3 and 7, immunized in this laboratory, was assayed previously in a similar fashion just after vaccine administration had been completed. All cultures were incubated at 37 C. in an upright position for 72 hours, after which they were terminated and the cells used in bacterial adherence and antigen stimulation assays described below.

**Bacterial Adherence Assays**

The first two culture vials, one containing PHA and the other plain, were centrifuged at 1500 rpm in an International clinical centrifuge for 5 minutes, the cell sediments washed three times with balanced Earle's solution, and resuspended in 2 ml. phosphate-buffered saline (PBS), pH 7.4. The plain and PHA-stimulated lymphocyte suspensions were both divided into four, 0.5 ml. aliquots. Individual 0.5 ml. portions were added to 2 ml. siliconized screw-top specimen bottles organized into 2 separate sets of 4 bottles each corresponding to the initial cell culture vials. Each group, then, the unprovoked lymphocytes and those exposed to PHA, was treated in the following manner. Two of the four specimen bottles received 0.5 ml. of a washed suspension of heat-killed, intact E. coli, standardized to an O.D. of 0.5 at 500 nm and the other two the same quantity and concentration of washed, formalinized, commercial polyvalent Salmonella vaccine. In addition, to determine the category (7S or 19S) of lymphocyte surface antibody, 1 bottle of the E.coli and Salmonella combinations in both sets received 0.25 ml. of 2-mercaptoethanol (2-ME) to make a final concentration of 0.2M. The tightly closed bottles were put on a variable speed rotator at room temperature for 30 minutes at 30 rpm. Separate wet coverslip preparations were made from each specimen bottle, giving a total of 8 wet mounts for each experiment, and examined under phase microscopy by complete scanning. The number and type of lymphocytes, on a percent basis, holding single or multiple organisms, were counted and used as the experimental endpoint. If a lymphocyte had reached a size of 15μ, and the nucleus had a vesicular, rather than clumped chromatin, with or without a nucleolus, it was called a blast cell and no longer was considered a small unchanged lymphocyte.

**Antigen Stimulation Assays**

Mitotic arrest in cultures three and four was produced by the addition of colchicine. The vial contents were centrifuged, the supernatants almost completely withdrawn, and multiple coverslip smears of the sediment stained with Wright's stain. These were scanned for the presence of mitoses which were considered to indicate an anamnestic immune response and represented the experimental endpoint.

**Auxiliary Mercaptoethanol Studies**

In order to compliment the bacterial adherence assays with mercaptoethanol, lymphocytes, after 72 hours in additional cell cultures, with and without PHA, but grown in the presence of a 14C-amino acid hydrolysate, were placed separately in plain PBS and that made 0.2M for mercaptoethanol. After one hour, the four supernatants in each experiment were removed, dialyzed for 72 hours against several changes of PBS, and the protein, presumably

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*Smith, Kline and French, Philadelphia, Pa.
†Difco, Detroit, Michigan.
‡Lederle Division American Cyanamid Company, Pearl River, N.Y.
*This grouping would, unavoidably, include small, intermediate and large blast forms.
†New England Nuclear Corporation, Boston, Mass.
**IMMUNOLOGIC MEMORY**

Table 1.—Experimental Summary of Lymphocyte Bacterial Adherence

<table>
<thead>
<tr>
<th>Donor No</th>
<th>Immun. With</th>
<th>Antigen</th>
<th>Plain †</th>
<th>Bacterial Adherence * With PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salm.</td>
<td></td>
<td></td>
<td>Unchanged</td>
</tr>
<tr>
<td>1</td>
<td>12 mos.</td>
<td>Salm.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E.coli</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>18 mos.</td>
<td>Salm.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E.coli</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>8 mos.</td>
<td>Salm.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E.coli</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Never</td>
<td>Salm.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E.coli</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Never</td>
<td>Salm.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E.coli</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>8 yrs.</td>
<td>Salm.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E.coli</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1 mo.</td>
<td>Salm.</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E.coli</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>3 mos.</td>
<td>Salm.</td>
<td>0</td>
<td>0</td>
<td>48</td>
</tr>
</tbody>
</table>

* Assayed as per cent lymphocytes with adherent organisms.
† Cultured without PHA.
‡ S = single organism adherent; M = multiple organisms adherent; Abs = no organisms adherent.

Discharged by the cells into the PBS, precipitated with 10 per cent trichloracetic acid. The precipitates were deposited by high-speed centrifugation, redissolved in 1N NaOH, and their radioactivity assayed in Bray’s solution with a Packard Tri-Carb scintillation spectrometer.*

*Bacterial Adherence

The outcome of the bacterial adherence assays are summarized in Table 1. When unstimulated cultured lymphocytes were incubated with E.coli, 5 to 16 per cent of these cells had single, adherent organisms (Fig. 1). These lymphocytes were morphologically similar to the ones immediately obtained

Fig. 1.—Bacterial adherence of unstimulated lymphocytes and E.coli. A single cell holds one organism (X 900).
from venous blood. The rare cell undergoing blastogenesis in the unprovoked cultures did not participate in bacterial adherence. Addition of PHA to the cultures resulted in blastoid transformation of 50 to 87 per cent of lymphocytes obtained from the 7 individual volunteers, and a complimentary number, in terms of per cent, remained unchanged.

The most pronounced alterations in bacterial adherence were observed with the lymphocytes which underwent blastogenesis. The total number of blast cells carrying E.coli varied from 11 to 50 per cent, and in all individual instances this final value was markedly increased over the per cent adherence with corresponding lymphocytes derived from the plain cultures (Table 1, E.coli as antigen). Moreover, whereas unprovoked small lymphocytes did not hold more than one organism, many blast cells from each PHA-stimulated culture had multiple bacilli attached to the cell surface (Fig. 2), inferring quantitatively elevated antibody synthesis, with the antibody having E.coli agglutinating properties. Nevertheless, an appreciable proportion of well-defined blast cells and morphologically unchanged lymphocytes were seen, as noted in Table 1, without adherent E.coli.

Bacterial adherence with Salmonella was overtly different from that performed using E.coli. Salmonella did not adhere to any of the lymphocytes from donors 1 to 6 (Table 1), whether the cells remained unprovoked or were exposed to PHA. Both small and blastoid lymphocytes were devoid of attached bacteria and many organisms were seen free-floating in the wet coverslip preparations. Unstimulated lymphocytes from donor number 7, who had been immunized most recently with vaccine, demonstrated bacterial adherence with Salmonella, indicating cellular antibody synthesis in a manner similar to that of a previous study in this laboratory. Exposure to PHA caused blastogenesis of the lymphocytes and a parallel, but less pronounced, increase in bacterial adherence as compared with the E.coli assays (Table 1). However, when this experiment was repeated 2 months later (3 months after immunization) bacterial adherence with Salmonella occurred neither with plain nor PHA-stimulated lymphocytes.

Incubation of plain lymphocytes with E.coli and 2-ME failed to significantly alter the bacterial adherence assays. In contrast, when PHA-stimulated lymphocytes were combined with E.coli in the presence of mercaptoethanol a
distinct difference was observed from tests not including 2-ME. Although the total number of these cells reacting in bacterial adherence remained the same, almost all the blast cells now held multiple organisms which, on occasion, completely covered the lymphoblast until only a portion of its vesicular nucleus remained visible. The small lymphocytes from the cultures with PHA behaved as did the cells from vials not containing this mitogen.

Addition of 2-ME to the assays using unstimulated lymphocytes and Salmonella prevented bacterial adherence with these organisms but did not change per cent adherence when PHA-stimulated lymphocytes were substituted for plain cells.

Antigen Stimulation Assays

Since a prior study indicated that no more than six mitotic figures developed spontaneously in vials without an in vitro antigenic stimulant, only cultures with more than this number of mitoses were considered significantly reactive. By scan, all cultures, done at various times, developed adequate mitotic figures when exposed to E.coli. A similar result was obtained immediately after immunization of patients 1, 2, 3, and 7 when their cells were provoked with Salmonella. However, when tested at the time of this particular protocol, Salmonella was not mitogenic for their cells under the conditions described. The same nonreactive outcome was obtained with lymphocytes from donors 4, 5, and 6 and from volunteer number 7 when tested at the three month interval.

Mercaptoethanol Incubations

These studies, summarized in Table 2, appeared to be concordant with the bacterial adherence assays, although total protein release rather than E.coli antibody was being measured by scintillation spectrometry. The amount of acid-precipitable radioactivity detected in the plain and mercaptoethanol-containing supernatants were not significantly different from one another when unprovoked lymphocytes were the protein source. Supernatant radioactivity rose appreciably when PHA-stimulated lymphocytes were placed in plain PBS after culture. This is in line with the increased circumferential adhesion of E.coli on such cells and infers that antibody and protein synthesis was enhanced and was detectably present on the lymphocyte surface. The greatest supernatant radioactivity, however, occurred when PHA-stimulated lymphocytes were incubated in 0.2M mercaptoethanol, an effect which parallels the greatest number of E.coli seen on the lymphocytes in the bacterial adherence system.

<table>
<thead>
<tr>
<th>Lymphocyte</th>
<th>Supernatant Radioactivity (cpm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
</tr>
<tr>
<td>Plain</td>
<td>4457</td>
</tr>
<tr>
<td>PHA-stimulated</td>
<td>18,840</td>
</tr>
</tbody>
</table>

* Mean values
DISCUSSION

The most important observation of this study was that PHA-stimulated lymphocytes reacted differently with E.coli and Salmonella in bacterial adherence implying that these antigens incite dissimilar forms and duration of immunity as defined by this immunologic procedure. PHA, in vitro, augmented lymphocyte bacterial adherence with E.coli in terms of the number of cells participating in this reaction and the quantity of bacteria circumferentially present on the cell surface. This effect was achieved mainly through blastogenic transformation of lymphocytes, although some morphologically unchanged cells in the PHA cultures, either as original elements not responding to the PHA, or as progeny of lymphocyte cell division taking place during culture, carried single microorganisms (Table 1).

There are several reasons to believe that bacterial adherence of E.coli to PHA-stimulated lymphocytes actually represents an antigen-antibody reaction closely analogous to anamnesis induced with a particular antigen, reflects immunologic memory stored within the cell, and is not dependent on the nonspecific or agglutinating influence of PHA itself. First, as shown in this and other laboratories, PHA does not agglutinate bacteria, hence bacterial adherence cannot be due to mixed agglutination of cells and bacilli. In addition, blastogenesis, per se, did not necessarily mean E.coli would attach to the cell surface. A considerable proportion of blasts (15–59 per cent, Table 1) did not hold bacteria, an observation consistent with the idea that antibody and nucleic acid synthesis need not be concordant. If adherence had been merely due to overall cellular transformation and resulting general increased synthesis of globulin having E.coli antibody characteristics all lymphoblasts might have participated in bacterial adherence. Blastogenesis and E.coli adherence took place, however, selectively. Finally, and probably of greatest significance, bacterial adherence with Salmonella was distinctly different from that with E.coli despite the use of lymphocytes from vaccine-immunized donors initially, but not finally, reactive directly to Salmonella antigen, and the occurrence of comparable blastogenesis in both assays. This outcome connotes differential antigenic recognition by morphologically similar lymphoblasts under the conditions prescribed.

The less pronounced and only transient reactivity of PHA-stimulated lymphocytes with Salmonella (donor 7, Table 1) compared with E.coli bacterial adherence, implies that the type, initiating means, and duration of contact of an antigen with antibody-producing cells may govern the establishment of permanent immunity. E.coli immunity, as shown in this laboratory, begins in utero or shortly after birth, is introduced transplacentally or via the gastrointestinal tract where the organisms remain, and is brought about with living bacteria to which lymphocytes are constantly exposed. This form of immunity is stable and is resistant to such agents as Actinomycin-D and x-irradiation, both of which are otherwise effective inhibitors of the immune response. Immunity to Salmonella, in contrast, is acquired, initiated deliberately with injection of a limited, fixed number of dead organisms, and is known to be short-lived. It is the E.coli form of natural immunity which may become an
inherent or at least transmissable portion of cellular components directing immunogenesis and, because of this, sensitive to stimulation by PHA. Differential mitogenicity of E. coli and Salmonella antigens as described here, ability of antilymphocyte serum to depress antibody titers to S. typhi, given as an overt immunogen in the dog, and not natural antibody to S. dysenteriae in the same animal, and the retention of reactivity to E. coli by human lymphocytes even when they have become leukemic, is additional experimental evidence which supports the concept outlined above.

Inability of mercaptoethanol to detectably alter E. coli bacterial adherence of unstimulated lymphocytes infers, at first glance, the major portion of immunologically reactive material on the cell surface belongs to the 7S rather than 19S class of immunoglobulins. This is in accord with these cells being participants in immunologic memory. Similarly, diminution of bacterial adherence with Salmonella in the presence of 2-ME suggests the small lymphocyte has on its surface 19S antibody representing a primary immune response. Yet, the unexpected enhancement of bacterial aggregation on PHA-stimulated lymphocytes, when bacterial adherence was performed with mercaptoethanol, could conceivably prevent definitive classification of surface antibody globulin as delineated by this reagent. Rather than indicating antibody type, under these circumstances, 2-ME may interfere with regulation of rapid antibody release by a PHA-stimulated cell which, in contrast to its unchanged counterpart, is metabolically very active, and depends on membrane stability for controlled antibody transfer. The occurrence of the highest degree of supernatant radioactivity in the PHA-mercaptoethanol incubations may indicate that disulfide-sulfhydryl interaction disturbed the integrity of the protein portion of the cell membrane and permitted inordinate protein release which was partly reflected by circumferential massing of E. coli in the bacterial adherence experiments.

The small lymphocyte, on the other hand, synthesizes antibody in a continuous but relatively indolent way and, therefore, its slow diffusion out of the cell is relatively unaffected by 2-ME. Neither decreased nor increased bacterial adherence with 2-ME, Salmonella, PHA-induced lymphoblasts, and surface antibody considered to be 19S in the small lymphocyte, is consistent with the idea that the dynamics of antibody release are not necessarily the same for every antigen and may be modified by the immunogenicity of the material inciting its synthesis.

The blastogeneic transformation of lymphocytes by phytohemagglutinin and their ultimate differential response in bacterial adherence with E. coli and Salmonella is compatible with the hypothesis stated earlier, that PHA acts on the cellular organelles which govern permanent immunity and define certain limits of lymphocyte immunologic recall. It is conceivable that antigen-antibody reactions, with recognized specificity, produced under the influence of phytohemagglutinin, indicate stability of a particular immune system and reflect differences between natural and acquired immunity.

**SUMMARY**

Using the technic of bacterial adherence, in which circumferential adhesion of microorganism to the lymphocyte surface indicates antibody production by
this cell, phytohemagglutinin (PHA)-stimulated lymphocytes reacted differently towards E. coli, a natural, lifelong immunogen, and towards Salmonella, one which is acquired and overtly given when the need arises. Bacterial adherence after cell culture was markedly augmented in terms of numbers of participating organisms and cells when PHA-provoked lymphoblasts, rather than small, unchanged lymphocytes, were incubated with E. coli. In contrast, this type of response either did not occur at all or only in a limited, transient fashion when Salmonella was substituted as the laboratory antigen despite the fact that the PHA-exposed lymphocytes were obtained from donors immunized with Salmonella and had reacted actively with E. coli. This differential antigenic recognition by PHA-induced lymphoblasts in bacterial adherence supports the theory that PHA, under the experimental conditions outlined, acts on cellular organelles which govern the permanency of immunity and reflects differences between the natural and acquired immune state.

SUMMARIO IN INTERLINGUA

In le empleo del technica de adherentia bacterial—in que adhesion circumferential de microorganismos al superficie lymphocytic indica le production de anticorpore per le lymphocytos—il esseva trovate que phytohemagglutinino-stimulate lymphocytos reageva differentemente con (1) Escherichia coli, que es un immunogeno natural de presentia pervital e (2) Salmonella, que es acquirit e administrate apertemente quando le necessitate se presenta. Adherentia bacterial post cytoculturation esseva marcatemente augmentate quanto al numeros del organismos e cellulas participante quando phytohemagglutinino-provocate lymphoblastos plus tosto que micre nonalterate lymphocytos esseva incubate con E. coli. Per contrasto, iste typo de responsa non occurreva del toto o occurreva solo de maniera restringite e transiente quando Salmonella esseva substituite como antigeno laboratorial, in despecto del facto que le lymphocytos exponite a phytohemagglutinina esseva obtenite ab donatores immunisate con Salmonella e que ille lymphocytos habeva reagite activemente con E. coli. Iste differentiante recognition antigenic per phytohemagglutinino-inducite lymphoblastos in adherentia bacterial supporta le theoria que phytohemagglutinina—sub le conditiones experimental delineate—age super organellas cellular que governa le permanentia del immunitate e reflecte differentias inter le statos de immunitate natural e acquirit.

ACKNOWLEDGMENT

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REFERENCES

5. Rabinowitz, Y., and Dietz, A.: Genetic control of lactate dehydrogenase and malate
IMMUNOLOGIC MEMORY

Immunologic Memory Of Phytohemagglutinin-Stimulated Lymphocytes

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