QUANTITATIVE STUDIES OF PHYTOHEMAGGLUTININ-INDUCED DNA AND RNA SYNTHESIS IN NORMAL AND AGAMMAGLOBULINEMIC LEUKOCYTES*

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Recent investigation of the lymphocytes of normal human subjects in vitro have shown that such cells undergo transformation and cytokinesis with concomitant synthesis of protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) when stimulated by antigens to which the subject has been previously sensitized (1-5). Staphylococcal products (3), specific antiserum to human leukocytes (5, 6), and streptolysin S (7) produce similar effects. Certain plant substances also have mitogenic properties, particularly phytohemagglutinin (PHA), an extract of the kidney bean Phaseolus vulgaris (8-14).

The mitogenic effect of PHA on lymphocytes can be determined quantitatively by measuring the amount of labeled precursor incorporated into RNA (13) and DNA (14). Since the basic defect in agammaglobulinemia appears to be located in the lymphocytes, their progenitors, or their progeny (15, 16), these assays were employed to investigate the lymphocytes of patients with acquired agammaglobulinemia. In the present study the PHA-induced response of leukocytes from patients with primary and secondary agammaglobulinemia was compared with the response of leukocytes from normal donors. The effect of sera from the agammaglobulinemic patients on PHA-initiated synthesis of RNA and DNA by normal leukocytes was also investigated.

Materials and Methods

Patients.—The study group consisted of nine adult patients with "acquired" agammaglobulinemia, all of whom were receiving monthly gamma globulin injections. [Infants with

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congenital agammaglobulinemia were not studied because the amount of blood required for assay (60 ml) seemed to contraindicate studies on infants.

Seven of the patients (No. 1-7) have “primary” agammaglobulinemia with serum immune globulin levels 2-10% of normal. All have had recurrent infections during a period of at least 5 yr; none of the seven has shown clinical or laboratory evidence of malignancy. Patients 8 and 9 have “secondary” agammaglobulinemia associated with lymphosarcoma and a thymoma, respectively. In patient 8 lymphosarcoma was diagnosed by laparotomy in 1951. Biopsy of jejunal and mesenteric lymph nodes in 1957 and of a subcutaneous deltoid nodule in 1963 showed changes typical of malignant lymphoma (reticulum cell type). From 1957 to 1963 the patient was treated elsewhere at various times with nitrogen mustard, irradiation, and chlorambucil. A serum protein electrophoretic pattern, first obtained in November 1962, showed severe hypogammaglobulinemia. Monthly gamma globulin injections were begun in July 1963. Since that time the patient has received no antitumor therapy and has remained asymptomatic. In patient 9, a diagnosis of thymoma and hypogammaglobulinemia was established in June 1964. Subsequently the thymoma has gradually increased in size. During the course of this study the patient was receiving 15 mg of prednisone daily. Whether the immunologic deficiency antedated the development of the associated disorders in these two patients is not known.

**Serum and Cell Preparations.**—Blood samples were obtained from each subject by venipuncture. A 20-ml aliquot was placed in a sterile tube and allowed to clot at 37°C without anticoagulant. The serum was removed aseptically.

Leukocyte suspensions were prepared from the remaining blood as described previously (14). Aliquots, 10 ml each, were added to each of four sterile 13-ml screw-cap centrifuge tubes containing 0.2 ml of heparin (heparin sodium, Upjohn Co., Kalamazoo, Mich., 1000 USP units/ml) and mixed by gentle inversion. Usually the cells were allowed to settle for 2 hr at room temperature; however, blood obtained from several patients with a very slow erythrocyte sedimentation rate, as is characteristic of agammaglobulinemia, was gently centrifuged. The leukocyte-containing supernatant plasma was removed. An aliquot was diluted with 3% acetic acid and the cell concentration determined with a hemocytometer. The desired volume of the plasma-cell suspension was centrifuged for 6 min. The cell pellet was resuspended in 5 ml of 0.05% sterile trypsin (Nutritional Biochemical Corp., Cleveland, Ohio) and incubated at 37°C for 7 min, during which the cells were gently dispersed several times with a pipette. The cell suspension was then centrifuged and the cell pellet resuspended in culture medium for planting. The final cell concentration was $1.0 \times 10^6$ per ml.

**Culture Medium.**—Eagle’s HeLa medium (EHM) (Colorado Serum Co., Denver, Colo.), modified by the addition of glycine (10$^{-3}$ M), serine (10$^{-2}$ M) and inositol (2 $\times$ 10$^{-3}$ M), was sterilized by passage through a Millipore filter, 0.22 $\mu$ pore size. Selas filtered sterile bovine serum (Colorado Serum Co.) or serum collected aseptically from normal or agammaglobulinemic subjects was added to the sterile EHM in a ratio of 1 part serum to 9 parts EHM. A single lot of bovine serum was used for all assays.

**Mitogen.**—Phytohemagglutinin-M (PHA-M) (Difco Laboratories, Detroit, Mich., lot No. 485836, 100 mg per vial) was used as the lymphocyte mitogen. The content of each vial was dissolved in 5 ml of sterile triple-distilled water; 1 ml of this stock solution was arbitrarily designated as possessing 1 unit of mitogenic activity. A fresh stock solution was prepared for each experiment; before use it was diluted tenfold with triple-distilled water so that 0.1 ml contained 0.01 unit of PHA.

**Assay Procedures.**—The assay systems for DNA (14) and RNA (13) are shown graphically in Fig. 1. Each assay was performed in duplicate. In each experiment cells from a normal donor were incubated in bovine serum, autologous serum, and serum from an agammaglobulinemic patient; cells from the agammaglobulinemic patient were incubated in bovine serum, autologous serum, and serum from the normal donor. The normal donor in each experiment was of
the same ABO and Rh blood group as the agammaglobulinemic patient, although varying these factors has had no apparent effect on leukocyte incorporation of labeled precursor (14, 17-19, footnotes 1 and 2). The normal donors were selected from a pool of volunteers in whom preliminary studies had shown a normal rate of incorporation of thymidine-2-14C into leukocyte DNA by the assay method used in this study. A control series of duplicate cultures in which 0.1 ml of distilled water was substituted for PHA-M were assayed simultaneously in each experiment. No incorporation was obtained in the absence of PHA-M.

**DNA Assay.**—Duplicate cultures of leukocytes were prepared by suspending approximately 3.0 × 10^8 cells in 3 ml of medium in 16 × 150 mm rubber-stoppered test tubes containing either 0.1 ml of the diluted PHA-M solution (i.e., 0.01 unit/culture) or 0.1 ml of triple-distilled water. All cultures were gassed with 5% CO₂ in air and incubated on a 4° slant at 37°C. After 48 hr (Fig. 1), 0.1 ml of EHM supplemented with 3 × 10⁻⁴ M amethopterin (methotrexate sodium, Lederle Laboratories, Pearl River, N. Y.) plus 1.5 × 10⁻⁵ M adenosine

![DNA ASSAY SEQUENCE](image)

![RNA ASSAY SEQUENCE](image)

Fig. 1. Schematic representation of assay systems for incorporation of labeled precursors into leukocyte DNA and RNA in phytohemagglutinin-stimulated leukocytes in vitro culture. TdR-2-14C, thymidine-2-14C; Cdr-H₃, tritiated cytidine; Ameth-A, amethopterin plus adenosine; PHA-M, phytohemagglutinin.

(Grade A, Calbiochem, Los Angeles, Calif.) (Ameth-A) was added to each culture. After further incubation for 16 hr, 0.1 ml of EHM containing 30 μg/ml of thymidine-2-14C (TdR-2-14C) (specific activity, 3.66 mc/mnmole, New England Nuclear Corp., Boston, Mass.) was added to each culture. Incubation was then continued for an additional 6 hr. After each addition, the cultures were gassed with 5% CO₂ in air before being returned to the incubator. Trypan blue staining showed that, on an average, 80-95% of the cells (both agammaglobulinemic and normal) were viable at the end of the culture procedure.

At the end of the 70 hr assay period, the cells were harvested and the acid-insoluble residue was obtained by successive treatment of the cells with 10% trichloroacetic acid, 80% ethanol, and a 1:1 mixture of absolute ethanol and ethyl ether. The acid-insoluble residue was dried, then dissolved in 0.5 ml of 90% formic acid. A 0.2 ml aliquot of each sample was added to 20 ml of ANPO (α-naphthylphenyloxazole, Packard Instrument Co., Downers Grove, Ill.) for

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3 The addition of Ameth-A blocks endogenous synthesis of thymidine and the concomitant synthesis of DNA. This effect is subsequently reversed in a synchronous manner by the addition of labeled thymidine (14, 20).
counting in a Packard Tri-Carb liquid scintillation spectrometer (21). The radioactivity in the residue obtained by this method is DNase sensitive (14).

**RNA Assay.**—Cultures were prepared in duplicate, as they were for the DNA assay, except that PHA was added not at the time of planting but after a 42 hr incubation period (Fig. 1). The cultures were incubated for 24 hr in the presence of PHA, then 0.1 ml of EHM containing 1.39 μg/ml of H\(^2\)-cytidine (specific activity 2.34 c/mmole, New England Nuclear Corp.) was added to each. After each addition the cultures were gassed with 5% CO\(_2\) in air. After further incubation for 2 hr, the cells were harvested. The acid-insoluble residue was prepared and the radioactivity counted as described for DNA. With the method used, the radioactivity in the acid-insoluble residue is releasable by RNAse (13). At the end of the culture period, 80-95% of both agammaglobulinemic and normal cells were viable as shown by trypan blue staining.

Tritiated cytidine rather than tritiated uridine was used for the RNA assay because the pool size of cytidine in mammalian cells is generally much smaller than that of uridine. McIntyre and Elbaugh (11) showed that RNA synthesis begins within the first 24 hr of exposure to PHA. The exact time curve of this period of RNA synthesis has been clarified by Mueller and Le
Mahieu (13), who have shown that RNA production increases exponentially as soon as PHA is added to the cells. DNA synthesis begins 30–36 hr after the addition of PHA, and this lag is independent of the time the cells have already been maintained in vitro. Thus, the incorporation of labeled cytidine into DNA under the conditions of these experiments is insignificant (13).

RESULTS

In the present study we measured the PHA-induced response of agammaglobulinemic cells cultured in vitro in medium containing bovine, normal, or autologous serum. In evaluating the results, incorporation of radioactivity into

![Graph showing effect of agammaglobulinemic serum on incorporation of labeled precursor into DNA and RNA of normal leukocytes.](image)

Fig. 3. Effect of agammaglobulinemic serum on incorporation of labeled precursor into DNA and RNA of normal leukocytes. Normal cells were incubated in Eagle's HeLa medium (EHM) supplemented with bovine serum or serum of each of nine agammaglobulinemic patients. The bars indicate the degree of incorporation of labeled precursor in cells cultured in EHM-agammaglobulinemic serum as compared with incorporation of the same cells in EHM-bovine serum (arbitrarily designated as 100). The degree of incorporation by cells in EHM supplemented with sera from normal human donors ranges from 80 to 140 in this assay system.

Incorporation of label by the cells of the agammaglobulinemic patients was considerably lower than that of normal leukocytes, regardless of which of the three types of serum was used to supplement the medium (Fig. 2).

In a separate series of experiments, the effect of sera from the agammaglobu-
linemic patients on incorporation of Tdr-2\(^{14}\)C and H\(^3\)-cytidine into normal leukocytes was compared with incorporation of label by normal cells suspended in bovine serum. As shown in Fig. 3, sera from four patients (No. 1, 2, 5, and 6) enhanced incorporation of Tdr-2\(^{14}\)C into leukocyte DNA, whereas sera from another four patients (No. 3, 4, 7, and 8) had no significant effect. Only one serum (patient 9) had an inhibitory effect on incorporation of label. Normal human sera neither significantly enhanced nor inhibited incorporation of Tdr-2\(^{14}\)C into DNA; the range of incorporation was 0.8 to 1.4 times that found with bovine serum. The various agammaglobulinemic sera had essentially the same effect on incorporation of labeled precursor into leukocyte RNA, except in the case of the patient with treated lymphosarcoma (No. 8). The serum of this patient enhanced incorporation of radioactivity into RNA but not into DNA.

In none of the experiments was there significant incorporation of labeled precursor into either DNA or RNA in the absence of PHA.

**DISCUSSION**

Because of its mitogenic properties, PHA is being widely used to investigate the function of the lymphocyte in health and disease (4–14, 22–27, footnotes 1 and 4). Recent studies with in vitro culture systems have shown that PHA is capable of stimulating rabbit lymph nodes to produce specific antibody (27) and human lymphocytes to synthesize gamma globulin (1) and specific antibody (2, 4, 10), although the latter is controversial. Circumstantial evidence suggests that some of the progeny arising after lymphocyte transformation and mitosis become plasma cells (28). Since the number of plasma cells (and hence their production of gamma globulin) is markedly decreased in agammaglobulinemia, data obtained in studies of the lymphocytes of such patients might provide an explanation for the failure of plasma cell genesis in this group of disorders and perhaps aid in elucidating the fundamental defect.

Theoretically, a serum factor capable of preventing transformation of lymphocytes into plasma cells might be responsible for the paucity of plasma cells in patients with agammaglobulinemia. This hypothesis seems unlikely since the sera of our patients with primary agammaglobulinemia had no inhibitory effect on the PHA-induced synthesis of DNA and RNA by normal human lymphocytes. The alternate explanation that the defect in primary agammaglobulinemia is located in the cells is supported by the decreased synthesis of DNA and RNA by agammaglobulinemic cells in the standard assay systems used in the present experiments. These observations are in accord with current data suggesting that primary acquired agammaglobulinemia is genetically determined and that the lymphocytes of such patients are defective (5, 16, 22–25, 29–36). Presumably, the genetically determined defect of the lymphocytes

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that precludes antigen-induced transformation of the cells into plasma cell precursors also precludes a normal biochemical response to PHA. The interpretations of the present findings are supported by a previous study from this laboratory, which demonstrated that lymphocytes from patients with primary agammaglobulinemia showed decreased incorporation of tritiated uridine into RNA after 48 hr of exposure to PHA, rabbit antiserum to human lymphocytes, or antigen (5). They are also in accord with the observation that although lymphocytes from patients with agammaglobulinemia appear capable of undergoing morphologic transformation when stimulated by PHA (24–26, 33), they synthesize less gamma globulin than do normal lymphocytes (4, 10, 23, 33). This synthetic defect is not unexpected in view of the decreased synthesis of gamma globulin in vivo in these patients (37–39). Whether the in vitro defect in DNA and RNA synthesis shown in the present experiments is also associated with an in vivo defect is conjectural. Such an association, however, might result in failure of cellular replication in response to antigen.

In contrast to the sera of the patients with primary agammaglobulinemia, the serum of the patient with secondary agammaglobulinemia and a slowly enlarging thymoma (No. 9) inhibited synthesis of both DNA and RNA in normal leukocytes. Sera from patients with active lymphopoietic tumors have been shown to inhibit PHA-induced synthesis of DNA in normal lymphocytes.1 Hence, the inhibitory effect of our patient's serum may only reflect tumor activity and have no relationship to the agammaglobulinemia. Alternatively, it may be attributable to corticosteroid therapy, which the patient was receiving at the time of the study; small doses of prednisone inhibit synthesis of DNA and RNA by PHA-stimulated leukocytes in culture (40) and therefore may do so in vivo.

In a previous study in this laboratory (5), synthesis of RNA by cells from patients with secondary agammaglobulinemia was not decreased during 48 and 72 hr of continuous culture. The apparent lack of accord with the results of the present study probably is related to differences in the method of assay. In the present experiments, incorporation of H3-cytidine was determined for a 2 hr period during the time of maximal RNA synthesis (Fig. 1) instead of during the total period of culture after the addition of PHA. Synthesis of RNA increases exponentially during the first 24 hr after the addition of PHA (13). Incorporation of H3-cytidine is therefore greater during the last 2 hr of the 24 hr period than during the total period of synthesis after the addition of PHA. The assay system used in the present experiments thus provides a more sensitive index of maximal RNA synthesis than the systems used previously in our laboratory and others. These results suggest the possibility that RNA synthesis in response to an antigen or a nonspecific stimulant such as PHA may be better sustained in secondary than in primary agammaglobulinemia.

In any event, the present data suggest that one or more defects are present
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within the lymphocytes of patients with acquired agammaglobulinemia. Both antigenic stimulation and PHA produce characteristic biochemical responses in normal cells in vitro. The lesser response of agammaglobulinemic cells in vitro suggests that a defective response to antigenic stimulation may also exist in vivo and may be the result of or associated with impaired cellular replication. Alternatively, the lymphocytes in agammaglobulinemia may be normal but unresponsive to such stimulation. According to a recent theory (15), the normal population of lymphocytes consists of two types of cells, morphologically undistinguishable but differing in ability to produce antibody. The normal lymphocytes of one type may be capable of responding to PHA or antigen, whereas the other type may be incapable of such response. The lymphocytes of agammaglobulinemic patients thus might consist entirely of a population of such unresponsive cells. This hypothesis seems unlikely since preliminary studies with this test system have demonstrated a partial metabolic defect in the cells of parents of patients with "acquired" agammaglobulinemia even though their serum gamma globulin levels were normal. This partial defect in the parents' cells indicates that acquired agammaglobulinemia is in reality a genetically determined disorder (41).

SUMMARY

Leukocytes from nine patients with acquired agammaglobulinemia were studied in vitro. Synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) induced by phytohemagglutinin was measured by determination of the degree of incorporation of labeled precursor. Synthesis of both DNA and RNA was decreased in the agammaglobulinemic cells. The presence of an inhibitor in the patients' sera could not be demonstrated. These results suggest that the basic defect in agammaglobulinemia is cellular rather than humoral.

BIBLIOGRAPHY


