A QUANTITATIVE STUDY OF THE STIMULATION OF DNA SYNTHESIS IN LYMPH NODE CELL CULTURES BY ANTI-LYMPHOCYTE SERUM, ANTI-GAMMA GLOBULIN SERUM, SPECIFIC ANTIGEN, AND PHYTOHEMAGGLUTININ*

By JOHN FOERSTER,‡ M.D., JEAN-PIERRE LAMELIN,§ M.D., IRA GREEN,‖ M.D., and BARUJ BENACERRAF,‖ M.D.

(*From the Department of Pathology, New York University School of Medicine, New York, 10016, and the Department of Hematology, Montefiore Hospital, New York 10467*)

(Received for publication 30 September 1968)

A diverse collection of substances is capable of stimulating the in vitro transformation of lymphocytes to large blast-like cells which resemble the pyroninophilic lymph node cells seen after antigenic stimulation in vivo (1). This morphological change is accompanied by increased protein (2), RNA (3), and DNA (4) synthesis and is followed eventually by mitosis of the transformed cells.

The agents capable of triggering this chain of events are many but may be considered under the broad headings of specific and general stimulants. The mitogenic activity of *specific stimulants* is characterized by its strict dependence on the prior sensitization of the cell donor to immunizing antigens, such as tuberculin (5), and protein-hapten conjugates (6). Usually stimulation by specific antigens involves only a small proportion of lymphocytes exposed to them in culture. The most characteristic feature of *general stimulants*, on the other hand, is their lack of dependence on prior sensitization and their related property of stimulating a majority of cells exposed to them in culture. General stimulants include phytohemagglutinin (7), pokeweed mitogen (8), staphylococcal filtrate (9), and antisera directed against immune globulins (10), or against determinants of the lymphocyte surface membrane (ALS) (11–13).

Blast transformation of lymphocytes therefore appears to be triggered either by an antigen-antibody reaction on the cell surface, or by an interaction of lymphocyte membrane receptors with a general stimulant. The mechanism by

* Supported by United States Public Health Service Grant AI 2094 and by the Health Research Council of the City of New York under contract I-138.

‡ Recipient of United States Public Health Service Special Fellowship 1F3 AI-35, 386-01.

§ Fellow of the Comité de Biologie Moléculaire, Paris, France.

‖ Present address: Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md. 20014

which these interactions stimulate the differentiation of lymphocytes is unknown. It was nevertheless felt that a comparative study of thymidine-^{3}H incorporation by sensitized lymphocytes in response to a wide range of concentrations of specific or general stimulants could provide information on the relative stimulating efficiency of these agents and on the properties, distribution, and homogeneity of the corresponding cellular receptors on the lymphocytes. The experiments to be presented show that only a fraction of lymphocytes from sensitized animals can respond to the immunizing antigen. The studies also suggest that sensitized lymphocytes bear specific antibody receptors of varying affinity since their response rises over a 10^6-fold increase in antigen concentration. In contrast, the maximal responses to ALS or phytohemagglutinin are considerably higher than those evoked by antigen. In addition the dose response curves with phytohemagglutinin and especially with ALS are very steep, indicating the greater homogeneity of the cellular components reacting with these agents.

In order to establish the relative sensitivity of lymphocytes to the various known properties of anti-lymphocyte sera, a comparative study of cytolysis, blast transformation, and immunofluorescent staining of lymph node lymphocytes by anti-lymphocytic sera was also carried out. A higher concentration of anti-serum is needed for blast transformation than for the other two reactions.

Materials and Methods

Reagents.—Eagle's minimum essential medium (MEM), penicillin, and glutamine were purchased from Grand Island Biological Laboratory, Grand Island, N. Y. Complete Freund's adjuvant (CFA) was prepared by adding killed *Mycobacterium tuberculosis* to incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) in a final concentration of 2 mg/ml. Bacto-phytohemagglutinin P (PHA) was also obtained from Difco Laboratories. The contents of each vial were dissolved in 5 ml of diluent, and tenfold serial dilutions of this stock solution were used in the studies described subsequently.

Fluorescein isothiocyanate was obtained from Baltimore Laboratories, Baltimore, Md. Tissue culture medium 199 (M 199) was purchased from Microbiological Associates, Bethesda, Md. Ficoll was obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. Sheep anti-rabbit γ-globulin was a gift from Dr. G. J. Thorbecke.

Hydroxide of hyamine 10X [p-(diisobutylcresoxyethoxyethol) dimethylbenzylammonium-hydroxide—1 molar solution in methanol], PPO [2–5 diphenyoxazole] and dimethyl POPOP [1,4-bis-2-(4 methyl-5-phenyloxazolyl)benzene] were all purchased from Packard Instrument Company, Inc., Downers Grove, Ill.

Preparation of Dinitrophenyl Guinea Pig Albumin (DNP-GPA).—Guinea pig albumin (GPA) was prepared by starch block electrophoresis as previously described (14). 2,4-dinitrofluorobenzene (DNP) (Eastman Organic Chemicals, Rochester, N. Y.), and GPA were allowed to react under alkaline conditions in a manner analogous to that outlined by Benacerraf and Levine (14). The resultant 2,4-DNP-GPA contained an average of 26 dinitrophenyl groups per molecule of GPA.

Preparation of Anti-lymphocyte Sera (ALS).—The four footpads of Hartley strain guinea pigs were injected with 0.1 ml of a saline emulsion of CFA. 10–30 days later the animals were anesthetized and exsanguinated from the heart. The enlarged popliteal, inguinal, and

axillary lymph nodes were removed, trimmed of fat, and teased apart in MEM. The cell suspension was filtered through gauze, centrifuged at 800 rpm for 10 min, and resuspended in normal guinea pig serum. The lymph node cells were then passed over a glass wool or glass bead column (15), yielding a cell population consisting almost entirely (>95%) of small lymphocytes. Approximately 10^8 cells were suspended in 1 ml of saline and emulsified in an equal volume of CFA. 0.5 ml aliquots of the emulsion were injected into each of the four footpads of New Zealand albino rabbits (Blue Spruce Farms, Altamont, N. Y.) weighing between 2.5 and 4.0 kg. 2 wk after this primary immunization the animals were boosted with similar cellular emulsions injected intramuscularly in several sites. Sera were collected between 2 and 4 wk after the booster immunization, decomplemented at 56° for 30 min, pooled, and stored at −20°C until used.

In an alternate immunization procedure albino rabbits received two intravenous injections of 10^9 column purified lymph node cells 2 wk apart. The animals were exsanguinated 7–10 days after the second injection, and the sera were decomplemented and stored as described previously. The in vitro assays yielded similar results for either method of ALS preparation.

Cytotoxicity Titers.—The cytotoxic properties of ALS for column purified lymph node cells were determined by an adaptation of the method described by Wigzell (16) for mouse isoantisera. Hemagglutination and hemolysin titers were measured by standard techniques.

Lymphocyte Cultures.—Guinea pigs were anesthetized with ether and exsanguinated from the heart. The axillary, inguinal, and popliteal lymph nodes were removed in sterile fashion, rinsed three times in MEM, and trimmed of fat. They were teased into a cell suspension in MEM containing glutamine, 10% decomplemented normal rabbit serum (NRS) and 50 units of penicillin per ml of medium. Large tissue aggregates were allowed to settle and the supernatant was centrifuged at 800 rpm for 10 min. The cell button was resuspended in the previously described culture medium and brought to a concentration of 2×10^7 nucleated cells per ml. 0.5 ml of this suspension (10^7 cells) was transferred to plastic culture tubes, 10 × 75 mm (Falcon Plastics, Los Angeles, Calif.), to which was also added 1.0 ml of culture medium containing the appropriate amount of stimulant (antigen, PHA, or ALS). The culture tubes were set up in triplicate for each dilution tested and incubated at 37°C in an atmosphere containing 5% CO_2. After 24 hr of culture 0.1 ml of a solution of tritiated thymidine (^{3}H-TdR; 10μc/20μg/ml) was added to each tube and the incubation was continued for an additional 24 hr. At this time cells were collected by centrifugation (4°C. 800 rpm. 10 min) and washed twice with 2.0 ml aliquots of phosphate buffered saline. 1.0 ml of cold 0.5 M perchloric acid (PCA) was added to the cell button and the resulting suspension was incubated at 4°C overnight. The precipitate was washed twice with cold 0.25 M PCA, once with cold methanol, and was then allowed to dry in air at 50°C. The dry residue readily dissolved in 0.5 ml of hyamine, and 0.2 ml of this solution was added to 15 ml of scintillant containing 5.0 gm of PPO and 100 mg of dimethyl POPOP per liter of toluene. The radioactivity was measured in a Tri-Carb liquid scintillation counter (Packard Instrument Company, Downers Grove, Ill.).

This method of determining the incorporation of ^{3}H-TdR into DNA was found to be as accurate and reproducible as the more time consuming technique of measuring the radioactivity of DNA hydrolysates which has been used in previous studies (6). Both of these procedures are superior to methods which simply rely on the measurement of total cellular radioactivity. In our preliminary investigations these latter techniques were found to yield high counts in control cultures, presumably because of a sizable intracellular ^{3}H-TdR pool not incorporated into DNA. This error does not significantly affect the results obtained from markedly stimulated cultures but does mask the lesser amounts of stimulation caused by weaker agents, such as very low concentrations of antigen.

Precipitin Curves.—Determinations of anti-gamma globulin antibodies in anti-lymphocyte

sera and anti-gamma globulin sera were made by the quantitative precipitin technique according to the method of Eisen et al. (17).

The rabbit anti-guinea pig γ_2-globulin and anti-F(ab')$_2$ sera were a gift of Dr. Victor Nussenzweig and were prepared as described earlier (18).

Absorption of ALS with Serum Proteins.—ALS was absorbed with normal guinea pig serum or guinea pig IgG according to the following schedule: to 20 ml of ALS was added 1.0 ml of a 1:20 dilution of normal guinea pig serum or 0.5 mg of IgG dissolved in 0.5 ml of saline. The mixtures were held at 4°C for 24 hr and then spun at 3000 rpm for 30 min in the cold. The clear supernatant serum was removed and reabsorbed several times in an identical manner. After four successive absorptions the sera were examined by immunoelectrophoresis and did not show (within the limits of the sensitivity of the method) antibodies against guinea pig serum proteins or IgG respectively.

Specific Absorption of ALS with Lymph Node Cells.—Lymph node cells were obtained from CFA injected guinea pigs as previously described and washed several times in MEM. ALS was mixed with cells in concentrations of 0.8–1.8 $\times$ 10^8 lymph node cells per ml of serum and held in ice for 30 min with frequent, gentle agitation. The mixtures were then centrifuged in the cold for 10 min at 800 rpm. A small aliquot of the cell absorbed serum was removed for testing and the rest was reabsorbed with a fresh batch of cells. The procedure was repeated several times.

Immunofluorescent Techniques.—

Preparation of fluorescein labeled sheep anti-rabbit γ-globulin antibody: The γ-globulin fraction of sheep anti-rabbit γ-globulin sera was isolated by double precipitation with 33% ammonium sulfate. This fraction was then conjugated with fluorescein isothiocyanate and purified on Sephadex and DEAE columns according to the technique of McDevitt et al. (19).

Preparation of cell smears: Guinea pigs previously immunized with CFA and protein antigens as part of another experiment were used as cell donors. Lymph nodes or thymic tissues were teased apart in M 199 containing 1% Ficoll. The cells were filtered through gauze, spun at low speed for 7 min, and washed once in M 199 containing 1% Ficoll. The cell button was resuspended in two drops of 5% Ficoll and smeared between two slides. After drying, the smears were fixed in 95% alcohol for 10 min at 25°C. The preparations were then washed several times in phosphate buffered saline (pH 7.2) before they were used in the fluorescent studies.

Lung macrophages were obtained by rinsing the tracheobronchial tree and alveoli with medium 199 containing 1% Ficoll. The cells harvested by this procedure were washed, smeared, and fixed as described above.

Preparation of sections: Wax sections were prepared according to the cold alcohol technique of Sainte-Marie (20). Prior to use, the sections were dewaxed in cold xylene, rehydrated through cold alcohol, and finally washed in pH 7.2 phosphate buffered saline.

Staining of smears or sections: Slides were covered with appropriate dilutions of ALS or normal rabbit serum for 30 min and were then washed extensively with phosphate buffered saline (pH 7.2). Subsequently the slide was flooded with fluoresceinated sheep anti-rabbit γ-globulin. After 30 min the preparation was washed repeatedly and mounted in buffered glycerol.

Parallel immunofluorescent studies were also carried out with living cells of lymph node or thymic origin. The method employed was that described by Cerottini and Brunner (21).

Microscopy and Photography.—The slides were examined with a Leitz Ortholux Microscope equipped with tungsten and Hb 200 UV light sources. The latter was used with a UG-1 exciter filter and a GG 13 barrier filter. Photography was performed with high speed Ektachrome film at exposure times of 30–90 sec. Corresponding control and experimental slides were always photographed in identical manner.

RESULTS

In Vitro Properties of Anti-Lymphocyte Sera (ALS)

Cytotoxic Properties of ALS.—The cytotoxic properties of rabbit antiguinea pig lymphocyte sera were evaluated 2 wk after primary immunization with guinea pig lymph node cells. Complement dependent cytotoxicity manifested by cellular disruption and release of intracellular chromium label was first noted at serum dilutions of 1/100 and increased logarithmically with increasing concen-

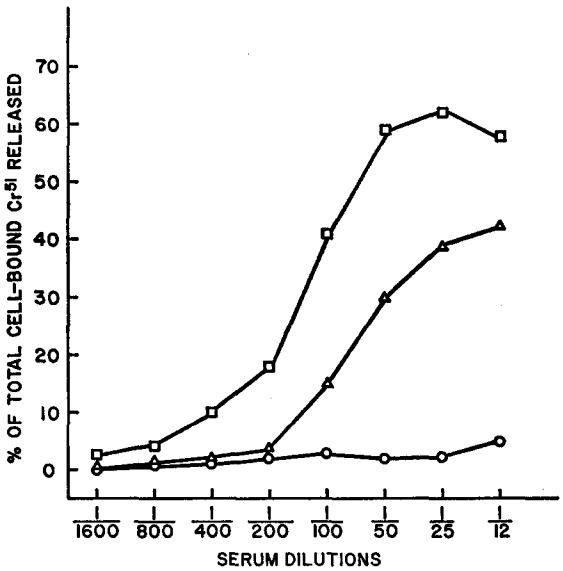


FIG. 1. Cytotoxicity titers of normal rabbit serum (O——O); ALS 14 days after primary immunization (△——△); and ALS 14 days after boosting (□——□). The cytotoxicity of ALS dilutions is expressed as the percentage of total lymphocyte-bound ^{51}Cr label released into the supernatant.

trations of anti-lymphocyte serum, the highest concentrations used exhibiting the most marked degree of cytotoxicity (Fig. 1). Normal rabbit sera exhibited no cytotoxicity, even in the highest concentrations tested. A booster injection of lymphoid cells given 2 wk after primary immunization resulted in a considerable increase in cytotoxicity, after which the cytotoxic properties of ALS showed a steady decline, even in the face of further booster injections. It is particularly noteworthy that absorption of ALS with normal guinea pig serum (or guinea pig IgG) to remove antiprotein-antibodies lead to no significant diminution of cytotoxicity (Fig. 2). In addition, rabbit anti-sera to guinea pig γ_2-globulin with high concentrations of antibodies (2.0 mg/ml) displayed a complete lack of cytotoxicity at all serum dilutions tested. It should be noted, that the absorp-

tion of ALS with lymph-node cells lead to progressive, and finally complete, loss of cytotoxicity.

Only low levels of hemagglutinin and hemolysin titers were noted in the sera of animals immunized with lymphocytes emulsified in CFA, the highest titer recorded for either assay never exceeding 1/32. Absorption of ALS with fresh guinea pig red cells (1 hr, 37°C) lead to complete loss of hemagglutinin and hemolysin activity, but only to insignificant changes in cytotoxicity titers.

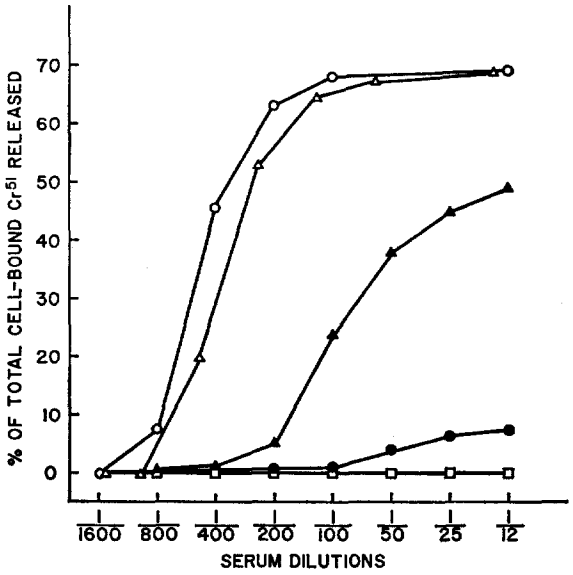


FIG. 2. Cytotoxicity titers of unabsorbed ALS (O——O), ALS absorbed with guinea pig serum (△——△), and ALS absorbed with 3.0×10^8 lymph node cells per ml of serum (▲——▲), or 5.2×10^8 lymph node cells per ml of ALS (●——●). Cytotoxicity titers of rabbit anti-guinea pig γ_2 globulin serum (□——□) having a specific antibody concentration of 2.0 mg/ml of serum are also shown. The cytotoxicity of the various sera is expressed as the percentage of the total lymphocyte-bound ^{51}Cr label released into the supernatant.

In Vitro Stimulation of Lymphocytes by ALS.—The striking effects of ALS on lymphocyte cultures in vitro are evident in Fig. 3: when lymph node cell cultures were exposed to ALS concentrations of 1% or less, no stimulating effects were observed. Once the threshold stimulating dose was reached, however, relatively small increments in the concentration of ALS stimulated a greatly increased incorporation of ^3H-TdR into cellular DNA. It is to be noted that maximal stimulation was seen with ALS concentrations only 5–10 times those of the highest nonstimulating dose. If the concentration of ALS was pushed beyond this optimal level, a decrease in ^3H-TdR uptake was invariably noted. No stimulation was seen with normal rabbit sera over the same dose range.

In Vitro Stimulation of Lymphocytes by Antigen.—It is of particular interest to compare the effect of ALS and antigen ($DNP_{26}GPA$) on lymphoid cells previously sensitized to this specific protein-hapten conjugate. The steep slope of the ALS dose-response curve stands in striking contrast to that obtained when comparable cultures are stimulated with varying concentrations of antigen (Fig. 3). Whereas ALS caused its maximal effect within a 5 to 10-fold increase in dose,

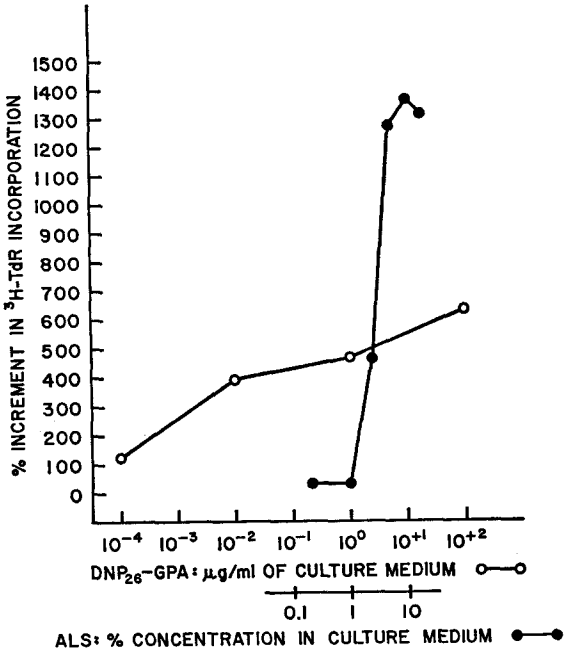


FIG. 3. The stimulation of in vitro ^{3}H-TdR incorporation into cellular DNA as a function of the concentration of $DNP_{26}GPA$ and ALS in lymph node cell cultures. ^{3}H-TdR incorporation is expressed as the percentage increment over that in control cultures derived from the same animal and incubated in the absence of antigen and ALS. Each curve represents the combined data of at least three individual experiments, each done in triplicate.

specific antigen showed progressive stimulation over a 10^6-fold increase in its concentration, resulting in the relatively flat slope of the dose-response curve. It should also be noted that the total number of counts incorporated into ALS-stimulated cultures was considerably greater than that incorporated maximally by antigen stimulated cultures.

In Vitro Stimulation of Lymphocytes by Phytohemagglutinin P.—The slope of the PHA dose-response curve is intermediate between that of antigen and ALS (Fig. 4). Whereas antigen showed progressive stimulation over a 10^6-fold increase in concentration and ALS reached peak activity within a 10-fold incre-

ment in dose, PHA developed a maximal response over a 10^3-fold increment in its concentration in culture medium. It is to be noted that optimal stimulation is restricted to a fairly narrow range, higher doses of PHA leading to a precipitous fall in the incorporation of ^{3}H-TdR. It is of interest that the total number of counts incorporated by maximally stimulated cultures is the same for ALS and PHA.

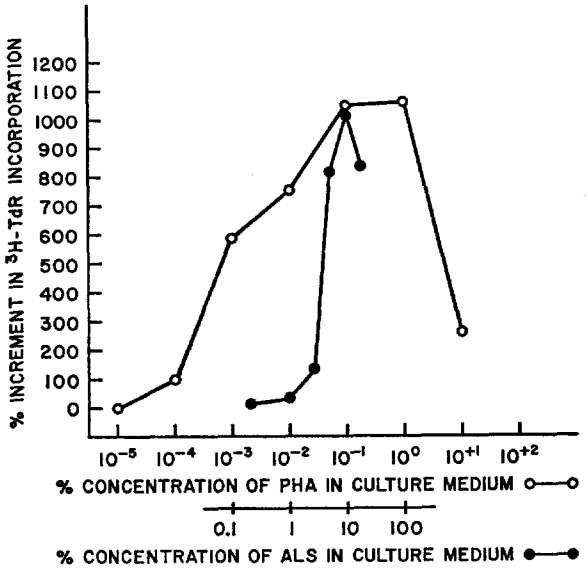


FIG. 4. The stimulation of in vitro ^{3}H-TdR incorporation into cellular DNA as a function of the concentration of PHA (O——O) and ALS (●——●) in lymph node cell cultures. ^{3}H-TdR incorporation is expressed as in Fig. 3. Each curve represents the combined data of at least four individual experiments, each done in triplicate.

Specificity of Anti-Lymphocyte Sera

Anti-gamma Globulin Activity of ALS.—The lymphocyte stimulating activity of anti-gamma globulin and antiallotype sera is well known (10). In order to control for the possibility that the mitogenic properties of our ALS might be due to antibodies against gamma globulins fortuitously contaminating the cellular preparations used for immunization, we examined our antilymphocyte sera for the presence of anti-guinea pig protein antibodies. We were also hoping to learn whether anti-lymphocyte sera might possibly have specificity for cell-bound gamma globulin, as has been suggested for antiallotype sera (10).

Immunoelectrophoresis of normal guinea pig serum developed with unabsorbed ALS revealed antibodies against many serum components including albumin and IgG. The concentration of the anti-IgG fraction of antibodies was

determined by the precipitin technique and was found to be 150 μg/ml of serum. However when ALS was absorbed with guinea pig serum or guinea pig IgG to the point of immunoelectrophoretic purity, no diminution of lymphocyte stimulating activity was noted (Fig. 5). Indeed, absorption of ALS with IgG alone lead to a perceptible increase in stimulation. The significance of this latter observation is not clear.

The Lymphocyte Stimulating Effect of Anti-Gamma Globulin sera.—The lymphocyte stimulating activity of rabbit anti-guinea pig gammaglobulin sera of two specificities was tested (Fig. 6). Although some stimulation was noted with high

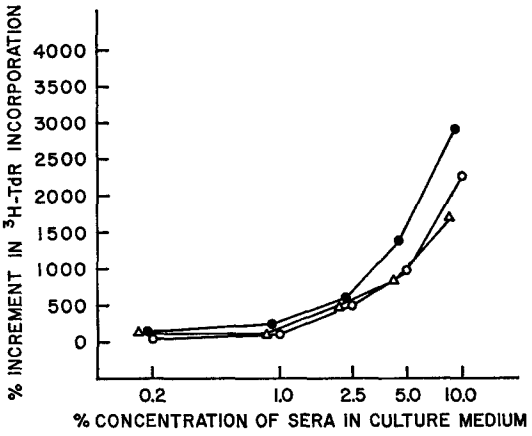


FIG. 5. The stimulation of in vitro ^{3}H-TdR incorporation into cellular DNA as a function of unabsorbed ALS (O——O), and ALS absorbed with guinea pig serum (△——△), or guinea pig IgG (●——●). ^{3}H-TdR incorporation is expressed as in Fig. 3. The results have been corrected for the dilution of ALS during the procedure of absorption. Each experiment was done in triplicate.

concentrations of anti-γ_2-globulin sera (20–100 μg/ml of culture medium), the amount of ^{3}H-TdR incorporation was small when compared to that seen with ALS. No stimulation was observed with anti-F(ab')$_2$ sera.

Absorption of ALS with Lymph Node Cells.—When ALS was absorbed with successive quantities of lymph node cells, a progressive decrease in the lymphocyte stimulating and lympholytic activities was noted (Fig. 7). It is of particular interest that the loss of cytotoxic properties of ALS occurred roughly in parallel with the loss of lymphocyte stimulating activity, although fewer cells were required for complete absorption of the latter. Cell-absorbed anti-lymphocyte sera which were no longer cytotoxic or stimulating at the concentrations tested, still had residual affinity for lymphocytes as revealed by immunofluorescent techniques, indicating that the concentration of anti-lymphocyte antibodies had simply been decreased to below threshold levels for the studies in

question. Cell-absorbed antisera still contained antibodies against guinea pig proteins, including IgG, as shown by immunoelectrophoresis.

The immunofluorescent studies demonstrated that small lymphocytes of lymph node (Fig. 8) or thymic origin (Fig. 9) stained brilliantly at all serum dilutions tested (1:10, 1:20, 1:100), whereas larger lymphocytes and macrophages exhibited fluorescence of less intensity. Cells treated with normal rabbit serum showed no detectable fluorescence (not illustrated). Absorption of ALS with

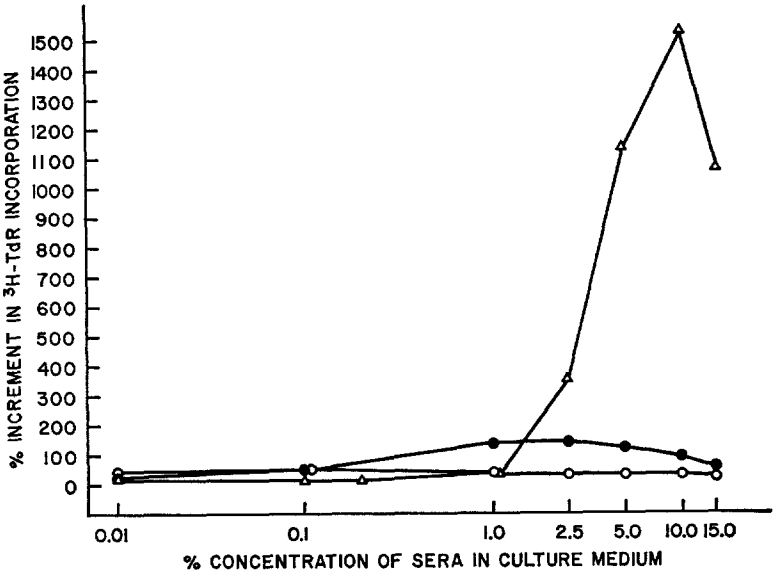


FIG. 6. The stimulation of in vitro ^{3}H-TdR incorporation into cellular DNA as a function of the concentration of ALS (△——△), anti-guinea pig γ_2 globulin serum (●——●), and anti-guinea pig F(ab')$_2$ serum (O——O). The latter two sera had undiluted antibody concentrations of 2.0 mg/ml of serum (●——●) and 2.3 mg/ml of serum (O——O) respectively. The results are expressed as in Fig. 3. Each curve represents the combined data of at least two experiments, each done in triplicate.

guinea pig serum did not alter the intensity of staining of lymphocytes but diminished considerably the amount of fluorescence displayed by macrophages. In contrast, absorption with 5×10^8 lymph node cells per ml of serum led to a considerable diminution in the degree of staining of lymphocytes, although under the experimental conditions used fluorescence was not totally abolished.

Cells obtained by pulmonary lavage showed staining of lymphocytes and macrophages only. Epithelial cells and erythrocytes never displayed fluorescence.

Sections of liver, kidney, and salivary glands showed some staining of interstitial areas and the lumina of blood vessels, although this fluorescence was com-

pletely abolished by prior absorption of ALS with guinea pig serum. The parenchymal cells of these organs never displayed discernible fluorescence, even at the highest serum concentrations tested.

When thymus or lymph node lymphocytes were stained in the living state, brightly fluorescent areas clearly separated by unstained zones became evident (Fig. 10). This is in contrast to the uniform fluorescence seen at the periphery of cells when fixed lymphocytes were stained (Figs. 8 and 9).

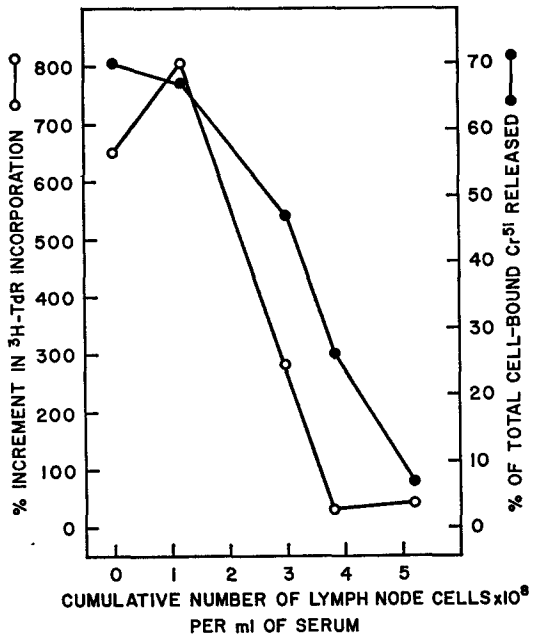


FIG. 7. DNA stimulation and cytotoxicity of ALS as a function of the cumulative number of lymph node cells used in the absorption of ALS. ^{3}H-TdR incorporation is expressed as the percent increment over control cultures stimulated by a 7.5% concentration of ALS in the culture medium. In previous experiments this concentration was found to give optimal stimulation. Cytotoxicity is expressed as the per cent of total lymphocyte-bound ^{51}Cr label released into the supernatant at the same concentration of ALS (7.5%).

DISCUSSION

The results of this study cast some light on the kinetics underlying the transformation of lymphocytes by different classes of mitogens and particularly highlight the differences between specific stimulants (antigens) on the one hand, and general stimulants (ALS and PHA), on the other. In addition the data indicate that the specificity of anti-lymphocyte antibodies is directed towards some membrane component other than cell bound gamma globulin.

Of particular interest is the comparison of the relative slopes of the dose-

response curves obtained when antigen sensitized cells are stimulated to transform by increasing concentrations of ALS or $DNP_{26}GPA$ (Fig. 3). The graded response over an exceedingly wide range of specific antigen concentrations has been noted previously (6) and is best explained by assuming the existence of a heterogeneous population of antigen-sensitized cells, each bearing on its surface a specific receptor of characteristic structure and binding affinity for antigen (6). Thus cells bearing high affinity receptors may be triggered with comparatively low concentrations of antigens, whereas cells bearing lower affinity receptors would require relatively high concentrations of antigen to be stimulated. This interpretation is further supported by the companion observation that guinea pigs immunized with low doses of antigen develop populations of lymph node cells which attain maximal in vitro stimulation at low concentrations of antigen, whereas cells from guinea pigs immunized with larger doses of antigen respond only minimally to low concentrations of antigen, considerably higher concentrations being needed for optimal response (6). In parallel with these observations are the findings relating the immunizing dose of antigen to the humoral antibody response:—large immunizing dose leads to the formation of antibodies of relatively low affinity, antibodies of progressively higher affinity being produced in response to decreasing doses of immunizing antigen (6) (22).

In striking contrast to the graded response of cell cultures to a wide range of antigen concentrations stands the steep slope of the dose-response curve obtained with ALS. A mere tenfold increase in the concentration of ALS encompasses the whole spectrum between complete lack of stimulation and maximal response, whereas PHA gives results intermediate between those of antigen and ALS.

If the thermodynamic principles thought to govern interactions between specific stimulants (antigens) and cell-bound receptors also govern the interaction between ALS and membrane-bound determinants, affinities of a rather homogeneous nature could be inferred for the latter reaction. This interpretation would however not take cognizance of the well-documented heterogeneity of the antibody response in terms of affinity for antigen, even when this is a hapten of well-defined structure and purity (23). A similar heterogeneity can be assumed to exist in the case of anti-lymphocyte antibodies. The clue to the difference in the kinetics of lymphocyte transformation by antigen and ALS must therefore be sought in the reversal of the roles of antigen and antibody whose interaction in either case triggers the series of events culminating in cell division. Thus, when lymphocytes are stimulated by antigen, a population of cells whose heterogeneity is determined by the binding affinities of its antibody-like receptors, competes for a rather homogeneous population of antigens and a graded dose-response curve results, reflecting the selective advantage of higher affinity cells in an environment of low antigen concentration. When, on the other hand, lymphocytes are stimulated by ALS, a heterogeneous population of antibodies competes for a rather homogeneous or at least randomly distributed set of antigens common to each cell. Under these circumstances, all cells capable of being stimulated have an

equal chance of binding antibody and the critical level of antibody necessary to trigger transformation is reached by all cells at similar concentrations of ALS. Basic to this interpretation is the concept that a critical number of antigen-antibody interactions are needed on each lymphocyte before transformation can be triggered. This concept finds some support in our immunofluorescent studies which show binding of anti-lymphocyte antibody to cells at concentrations which do not result in stimulation.

The nature of the interaction between PHA and cellular determinants is largely unknown, although certain observations are of interest. It is well recognized, for instance, that many plant agglutinins show exquisite specificity for well-defined cellular determinants, notably blood group substances (24). Other agents such as concanavalin A, a phytohemagglutinin derived from the Jack bean (*Canavalia ensiformis*) possess the unique property of forming precipitates with certain types of branched α-glycans (25) in addition to being excellent hemagglutinins and general mitogenic stimulants (26). This particular agent gives rise to precipitin-like curves when it is allowed to react with specific polysaccharides, and the reaction may be quantitatively inhibited by low molecular weight carbohydrates ("haptens") sharing certain structural characteristics, suggesting a close analogy to the antibody-antigen system (27). If the interaction between PHA and the cell surface is of a similar nature and an interaction of this type is indeed important in initiating mitogenesis, then the binding of PHA to cellular determinants could be considered to involve affinities of a more restricted nature than those thought to govern interactions between antigen and sensitized cells.

The nature of the membrane determinant which reacts with anti-lymphocyte antibody is obscure. Sell and Gell (10) have extensively investigated the stimulation of rabbit lymphocytes by antiallotype sera and have concluded, largely on the basis of the strict allotypic specificity of the reaction, that antigenic determinants of a specific gamma globulin allotype must be present on or in the cell in order for the anti-allotype serum to be effective. The stimulation of lymphocytes by ALS, however, is unlikely to involve antibody molecules of the ordinary variety acting as cellular determinants, as extensive absorption of ALS with guinea pig IgG or whole guinea pig serum results in no loss of lymphocyte stimulating activity. In addition, sera raised in rabbits against guinea pig γ_2-globulin or its $F(ab')_2$ fragment with high concentrations of antibody against these proteins lead to a much smaller stimulation of lymphocytes than ALS or even specific antigen. On the other hand, the immunofluorescent and cellular absorption studies reported here as well as those of others (28, 29) strongly suggest that anti-lymphocyte sera have specificity for antigenic determinants restricted largely to lymphocytes and absent from other tissues variously tested (red cells, salivary glands, liver, kidney, and bronchial epithelium).

The amount of DNA synthesis stimulated by anti-IgG antibodies in our experiments is comparable to the limited incorporation of ^{3}H-TdR observed by Sell, and Sell and Gell, for sheep and guinea pig anti–rabbit γ-globulin sera (30) (10). The striking mitogenic efficiency of antiallotype sera, on the other hand, is quantitatively comparable to the degree of stimulation observed with ALS or PHA in our studies. The reason for the greater effectiveness of anti-allotype sera as compared to anti-γ-globulin sera is not clear, but may be related to the more restricted antigenic specificity

of the former. It is also of interest that the mitogenic capacity of antiallotype sera is readily absorbed with relatively small numbers of allotype-specific cells (4×10^7/ml of serum), and that incubation of cells and antiallotype serum for only 15 min leads to significant stimulation of lymphocytes (10), both suggesting that antibodies of rather high affinity are involved.

The discretely patchy fluorescence observed when ALS-treated living cells are stained with labeled anti-rabbit IgG is of considerable interest. Identical findings were described by Cerottini and Brunner (21) who studied the distribution of mouse isoantigens and concluded that isoantigenic determinants of normal and tumor cells were concentrated in discrete areas of the cell surface. Our studies attest to the patchy distribution of antigens recognized by heterologous anti-lymphocyte sera, but do not allow detailed speculation as to their nature.

The cellular reactions underlying the stimulation of lymphocytes by ALS and PHA remain unknown. The inability to separate the leuko-agglutinating from the mitogenic activity of PHA has in the past led to the conclusion that agglutination is a necessary prerequisite to stimulation (31). The leukocyte stimulating activity of ALS likewise resides only in the agglutinating whole gamma globulin molecule or its $F(ab')_2$ fragment, while the monovalent Fab' molecule lacks both agglutinating and stimulating activity (32, 35). Sell has recently shown, however, that Fab' fragments of sheep antibody to rabbit IgG are capable of eliciting a very limited mitogenic response as evidenced by a twofold increase of ^{14}C thymidine uptake in lymphocytes stimulated with optimal concentrations of Fab' (33). It need hardly be stressed that agglutination and stimulation may be companion phenomena independent of each other. Indeed it has recently been demonstrated that lymphocytes coated with Vi polysaccharide can no longer be agglutinated but are nevertheless readily stimulated by PHA (34). It should also be noted that many other general stimulants are non-agglutinating, notably antiallotype sera (10), and staphylococcal filtrate (9).

It is of interest that the lymphocyte stimulating activity of ALS is readily removed by absorption with lymph node cells. Thus at least a tenfold reduction in the effective concentration of anti-lymphocyte antibody must have been brought about by exposing 4×10^8 lymphocytes to 1 ml of serum. Although the cytotoxic properties of absorbed sera diminished roughly in parallel with the loss of stimulatory power, 4×10^8 lymphocytes per ml of serum were insufficient to abolish cytotoxic manifestations completely. The parallel decrease of cytotoxic and stimulating properties does suggest, however, that the antibodies involved in each case are of similar affinity. Both activities may indeed be mediated by the same antibody, leading to stimulation in the absence of complement and to lysis in its presence. If this is so, complement dependent cell lysis requires fewer antibodies than cellular stimulation, as ALS concentrations which no longer cause stimulation still lead to effective cell lysis in the presence of complement.

These studies only provide limited clues concerning the immuno-suppressive action of ALS in vivo. Nevertheless it seems clear that direct stimulation of lymphocytes cannot play a major role, as relatively high doses of ALS are re-

quired to bring about stimulation and it is unlikely that effective concentrations of ALS could be achieved and maintained in vivo. Even more compelling is the observation that ALS is always administered in the presence of endogeneous complement which efficiently leads to lysis of antibody-coated lymphocytes, even at antibody concentrations which are too low to result in stimulation.

SUMMARY

Rabbit anti-guinea pig lymphocyte serum is an efficient stimulus of the synthesis of DNA by guinea pig lymph node cells in vitro. The ability of ALS to stimulate lymphocytes is characterized by its lack of dependence on prior sensitization, the magnitude of the response it elicits, and the stimulation of all sensitive lymph node cells simultaneously within a very narrow range of ALS concentrations. In contrast to this homogeneous response to ALS, the stimulation of lymph node cells by antigen proceeds in graded fashion over a wide range of concentrations, thus reflecting the heterogeneity of the response of sensitized cells to antigen. PHA gives a response which is intermediate between that of ALS and antigen.

ALS appears to have specificity for membrane determinants shared by lymphocytes but not found on other tissues. This specificity does not involve cell-bound gamma globulin. The serum activity mediating lymphocyte stimulation as well as cytotoxicity is readily removed by absorption with lymph node cells.

BIBLIOGRAPHY

1. Oppenheim, J. J. 1968. Relationship of in vitro lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. *Fed. Proc.* **27**:21.
2. McIntype, O. R. and F. G. Ebaugh Jr. 1962. The effect of phytohemagglutinin on leukocyte cultures as measured by P^{32} incorporation in DNA, RNA and acid soluble fractions. *Blood.* **19**:443.
3. Cooper, H. L., and A. D. Rubin. 1965. RNA metabolism in lymphocytes stimulated by phytohemagglutinin: Initial responses to phytohemagglutinin. *Blood.* **25**:1014.
4. MacKinney, A. A., Jr., F. Stohlman, Jr., and G. Brecher. 1962. The kinetics of cell proliferation in cultures of human peripheral blood. *Blood.* **19**:349
5. Permain, G., R. R. Lycette, and P. H. Fitzgerald. 1963. Tuberculin-induced mitosis in peripheral blood leukocytes. *Lancet.* **1**:637.
6. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1968. Specificity of cellular immune responses. Antigen concentration dependence of stimulation of DNA synthesis in vitro by specifically sensitized cells, as an expression of the binding characteristics of cellular antibody. *J. Exp. Med.* **127**:25.
7. Nowell, P. C. 1960. Phytohemagglutinin: An initiator of mitosis in cultures of normal human lymphocytes. *Cancer Res.* **20**:462.
8. Chessin, L. N., J. Borjeson, P. D. Welsh, S. D. Douglas, and H. L. Cooper. 1966. Studies on human peripheral blood lymphocytes in vitro. II. Biochemical

studies on the transformation of lymphocytes by pokeweed mitogen. *J. Exp. Med.* **125**:873.

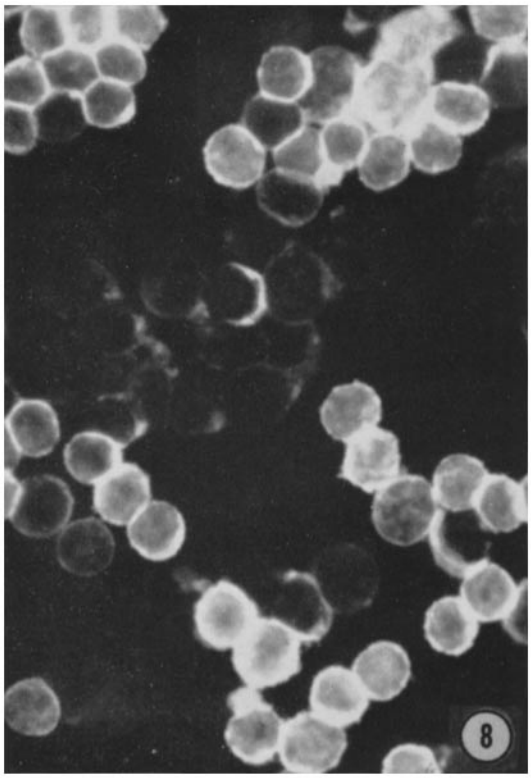
9. Ling, N. R., E. Spicer, K. James, and N. Williamson. 1965. The activation of human peripheral lymphocytes by products of staphylococci. *Brit. J. Haemat.* **11**:421.
10. Sell, S., and P. G. M. Gell. 1965. Studies on rabbit lymphocytes in vitro. I. Stimulation of blast transformation with an antiallotype serum. *J. Exp. Med.* **122**:423.
11. Gräsbeck, R., C. Nordman, and A. De la Chapelle. 1963. Mitogenic action of antileukocyte immune serum on peripheral leukocytes in vitro. *Lancet.* **2**:385.
12. Holt, L. J., N. R. Ling, and D. R. Stanworth. 1966. The effect of heterologous antisera and rheumatoid factor on the synthesis of DNA and protein by human peripheral lymphocytes. *Immunochemistry.* **3**:359.
13. Woodruff, M. F. A., B. L. Reid, and K. James. 1967. Quantitative studies with antilymphocyte antibody. *Nature.* **216**:758.
14. Benacerraf, B., and B. B. Levine. 1962. Immunological specificity of delayed and immediate hypersensitivity reactions. *J. Exp. Med.* **115**:1023.
15. Rabinowitz, Y. 1964. Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass bead columns, including tissue culture observations. *Blood.* **23**:811.
16. Wigzell, H. 1965. Quantitative titrations of mouse H2 antibodies using Cr^{51} labelled target cells. *Transplantation.* **3**:423.
17. Eisen, H. N., M. E. Carsten, and S. Belman. 1954. Studies of hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl group as a determinant in the precipitin reaction. *J. Immunol.* **73**:296.
18. Nussenzweig, V., and B. Benacerraf. 1966. Presence of identical antigenic determinants in the Fd fragments of γ_1 and γ_2 guinea pig immunoglobulins. *J. Immunol.* **97**:171.
19. McDevitt, H. O., J. H. Peters, L. W. Pollard, J. G. Harter, and A. H. Coons. 1963. Purification and analysis of fluorescent labelled antisera by column chromatography. *J. Immunol.* **90**:634.
20. Sainte-Marie, G. 1962. A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* **10**:250.
21. Cerottini, J. C. and K. T. Brunner. 1967. Localization of mouse isoantigens on the cell surface as revealed by immunofluorescence. *Immunology.* **13**:395.
22. Eisen, H. N., and G. W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry.* **3**:996.
23. Eisen, H. N. 1964–1965. The immune response to a simple antigenic determinant. *Harvey Lect.* **60**:1.
24. Boyd, W. C. 1963. The Lectins: their present status. *Vox Sang.* **8**:1.
25. Goldstein, I. J., C. E. Hollerman, and J. M. Merrick. 1965. Protein-carbohydrate interaction. I. The interaction of polysaccharides with concanavalin A. *Biochim. Biophys. Acta.* **97**:68.
26. Krüpe, M., W. Wirth, D. Nies, and A. Ensgraber. 1968. Untersuchungen uber die mitogene Wirkung agglutininhaltiger Extrakte aus verschiedenen Pflanzen auf die kleinen Lymphozyten des peripheren Blutes in vitro. *Z. Immunitaetsforsch., Allergie Klin. Immunol.* **135**:19.

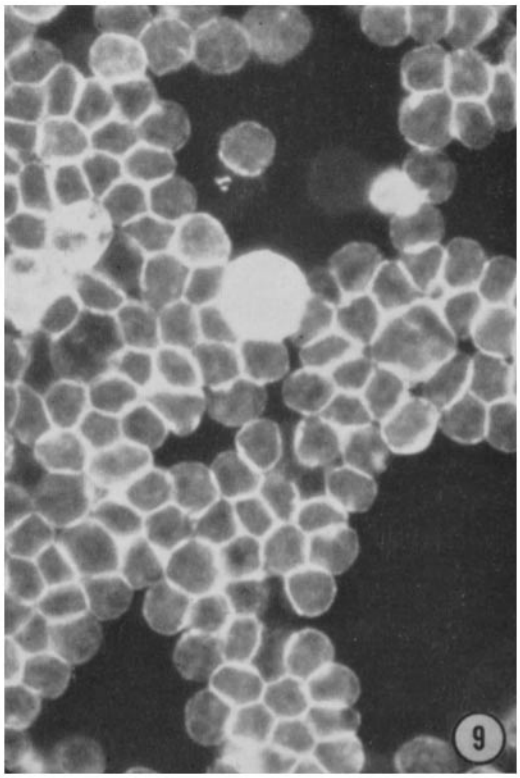
27. So, L. L., and I. J. Goldstein. 1967. Protein carbohydrate interaction. IX. Application of the quantitative hapten inhibition technique to polysaccharide-concanavalin A interaction. *J. Immunol.* **99:**158.
28. Gray, J. G., A. P. Monaco, M. L. Wood, and P. S. Russell. 1966. Studies on heterologous antilymphocyte serum in mice. I. In vitro and in vivo properties. *J. Immunol.* **96:**217.
29. Potworowski, E. F., and R. C. Nairn. 1968. Lymphoid-specific antigens: distribution and behaviour. *Immunology.* **14:**591.
30. Sell, S. 1967. Studies on rabbit lymphocytes in vitro. VI. The induction of blast transformation with sheep antisera to rabbit IgA and IgM. *J. Exp. Med.* **125:**393.
31. Nordman, C. T., A. De la Chapelle, and R. Gräsbeck. 1964. The interrelations of erythroagglutinating, leukoagglutinating and leukocyte mitogenic activities of Phaeseoleus Vulgaris phytohemagglutinins. *Acta Med. Scand. Suppl.* **412:**49.
32. Woodruff, M. F. A., B. Reid, and K. James. 1967. Effect of antilymphocytic antibody and antibody fragments on human lymphocytes in vitro. *Nature.* **215:** 591.
33. Sell, S. 1967. Studies on rabbit lymphocytes in vitro. VII. The induction of blast transformation with $F(ab')_2$ and Fab′ fragments of sheep antibody to rabbit IgG. *J. Immunol.* **98:**786.
34. Borjeson, J., L. N. Chessin, and M. Landy. 1967. Dissociation of leukagglutinating and transforming properties of phytohemagglutinin by coating of lymphocytes with Vi polysaccharide. *Int. Arch. Allergy Appl. Immunol.* **31:**184.
35. Riethmüller, G., D. Riethmüller, H. Stein, and P. Hansen. 1968. In vivo and in vitro properties of intact and pepsin-digested heterologous anti-mouse thymus antibodies. *J. Immunol.* **100:**969.

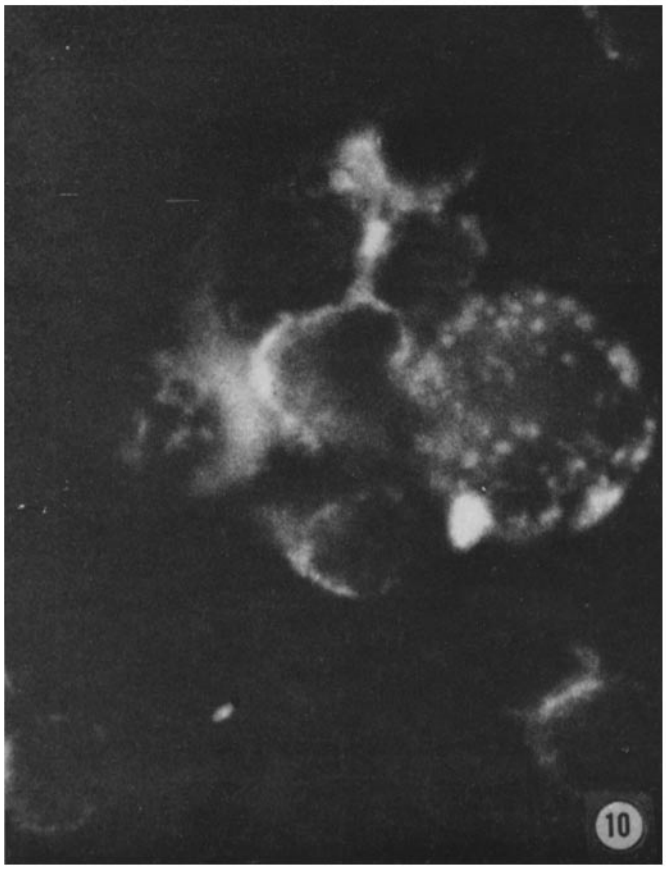
FIG. 8. Smear of dissociated guinea pig lymph node cells exposed to ALS in vitro and counterstained with fluorescein labeled sheep anti-rabbit gamma globulin serum. × 450.

FIG. 9. Smear of dissociated guinea pig thymus cells exposed to ALS in vitro and counterstained with fluorescein labeled sheep anti-rabbit gamma globulin serum. × 450.

FIG. 10. Lymph node lymphocyte exposed to ALS in the *living state* and counterstained with sheep anti-rabbit gamma globulin. × 980.


8


9


10