STUDIES ON RABBIT LYMPHOCYTES IN VITRO

III. Protein, RNA, and DNA Synthesis by Lymphocyte Cultures after Stimulation with Phytohaemagglutinin, with Staphylococcal Filtrate, with Antiallotype Serum, and with Heterologous Antiserum to Rabbit Whole Serum*

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(Received for publication, June 1, 1965)

In a previous report it was shown that the peripheral lymphocytes of rabbits could be stimulated *in vitro* to transform into blast cells and to synthesize DNA if cultured in the presence of heterologous antisera to rabbit IgG, of specific antiallotype sera, of phytohaemagglutinin, or of staphylococcal filtrate (1). In the present report it will be demonstrated that antisera to rabbit serum proteins other than IgG are also effective in the production of blast transformation. Others have demonstrated that peripheral lymphocytes of human origin when transformed *in vitro* will synthesize protein (2, 3), RNA, and DNA (4–8). However, the relationship between the synthesis of these products is poorly understood. The present report describes experiments designed to demonstrate the synthesis of protein and nucleic acids by stimulated rabbit lymphocytes and to define the relationship between the synthesis of these products and blast cell formation. In addition, since it has been claimed that peripheral lymphocytes of human origin specifically synthesize immunoglobulins when stimulated with phytohaemagglutinin or specific antigen (2, 3), an attempt is made to characterize the proteins made by stimulated rabbit blast cells. The results indicate that there is a sequence of events set off by all transforming agents consisting of the synthesis of protein followed, in turn, by RNA synthesis, histologic blast transformation, DNA synthesis, and finally, mitosis. Stimulated cells synthesize little, if any, γ-globulin (IgG) and it is suggested that the majority of products synthesized by stimulated cells are ultimately related to the capacity of these cells to divide.

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* This investigation was supported in part by a Public Health Service fellowship (1-F3-A1-23,308-01) from National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

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Materials and Methods

Lymphocyte Cultures.—The lymphocytes were separated from the peripheral blood of healthy adult rabbits and cultured *in vitro* as described in a previous report (1). An exception to the usual bleeding procedure (ear vein) was required in some experiments to obtain a sufficient number of lymphocytes from one animal. In such situations blood was obtained by heart puncture after nembutal anesthesia. Lymphocyte cultures were stimulated with phytohaemagglutinin (PHA), staphylococcal filtrate (SF), and antiallotype serum (anti-As4), or a sheep antiserum to rabbit whole serum (Anti-WRS) as previously described, (1).

Protein, RNA, and DNA Synthesis.—To determine the relationship between protein synthesis, RNA synthesis, DNA synthesis, and blast transformation in lymphocyte cultures, a lymphocyte suspension was prepared from one rabbit and the subsequent cultures divided into three groups. The first group consisted of 20 cultures containing 1×10^6 lymphocytes per culture. The first five cultures of this group were grown in the presence of normal serum (control), the second five cultures in normal serum plus PHA, the third five in normal serum plus SF, and the last five in antiallotype (As4) serum. At 12, 24, 48, 72, and 96 hours one from each subgroup was centrifuged, the button of cells obtained smeared on a glass slide, and the smears stained with Jenner-Giemsa. These smears were then evaluated for per cent blast transformations by light microscopy. The second group, consisting of 20 cultures containing 5×10^6 leucocytes each, grown in lysine-free Eagle's medium, was subdivided in four groups of four cultures each (control, PHA, SF, and anti-As4 serum). One culture in each subgroup was centrifuged at 12, 24, 48, and 72 hours, the button of cells obtained was washed once in 0.85 per cent NaCl, dried, and dissolved in hyamine (Nuclear Enterprises, Edinburgh, Scotland). Twelve hours prior to processing of the 12 and 24 hour cultures and 24 hours prior to processing of the 48 and 72 hour cultures 0.1 μc of C^{14}-lysine (The Radiochemical Centre, Amersham, England, Batch 61), was added to each culture. The hyamine cell solution was transferred to a counting bottle with 10 ml phosphore solution and the radioactivity present in each bottle determined as described previously (1).

The third group of cultures, consisting of 24 cultures containing 5×10^6 leucocytes each, grown in regular medium, was subdivided into four groups (control, PHA, SF, and anti-As4) of six cultures each. At 12, 24, 36, 48, 72, and 96 hours one culture of each subgroup was centrifuged, the button of cells obtained washed once in 0.85 per cent NaCl, washed twice in 5 per cent trichloracetic acid (TCA), and the RNA and DNA extracted by a modification of the Schmidt-Tannhauser technique (9). Twelve hours prior to the processing of the 12, 24, 36, and 48 hours cultures and 24 hours prior to the processing of the 72 and 96 hours cultures 5 μc of p^{32} (The Radiochemical Centre), were added to each culture bottle. After separation of the RNA and DNA of each culture the amount of radioactivity in each fraction was determined in a liquid Geiger-Muller tube using a round lead castle and an automatic scaler (type N610, EKCO Electronics Ltd., London).

Groups identical to those described above were set up using normal sheep serum and sheep anti-WRS (368/V). This anti-WRS serum was produced by immunising sheep with whole rabbit serum and is polyvalent with regard to rabbit serum proteins.

Autoradiographs.—One μc of tritiated thymidine or 1 μc of tritiated cytidine (The Radiochemical Centre), was added at 0, 6, 24, and 48 hours to four groups of lymphocyte cultures (control, PHA, SF, and anti As4) with four cultures in each group containing 1×10^6 leucocytes per culture. The respective cultures were centrifuged at 6, 24, 48, and 72 hours, and the button of cells obtained from each culture was smeared on a glass slide. Autoradiographs were made from these smears by layering with Kodak AR 10 stripping film and exposing and processing as described previously (1).

Radiolabelled Cell Protein Production.—To obtain radiolabelled proteins synthesized by rabbit lymphocytes *in vitro* a series of experiments in which lymphocytes were cultured in

lysine-free Eagle's medium in the presence of C^{14}-lysine were performed. Each culture contained 5×10^6 leucocytes, 3.0 ml lysine-free Eagle's medium and either (*a*) 0.5 ml normal rabbit serum (control), (*b*) 0.5 normal rabbit serum plus stimulant (PHA or SF), (*c*) 0.5 ml sheep anti-WRS, or (*d*) 0.5 ml anti-As4 serum. No attempt was made to remove the lysine present in the various sera, so that some differences in the amount of C^{14}-lysine incorporated into protein by the various cultures might be expected due to different amounts f lysine in the sera used. To obtain the labelled proteins produced by the five groups of cultures at different time intervals during *in vitro* culture C^{14}-lysine was added and the cultures processed according to the following schedule: (*a*) 0.1 μc of C^{14}-lysine was added to one set of cultures at 0 hours and the cells from these cultures processed at 24 hours; (*b*) C^{14}-lysine was added at 24 hours and the cells from this set of cultures processed at 48 hours. Th cells from each of four cultures (total 20×10^6 lymphocytes) maintained under the same xperimental conditions were pooled, washed once with 0.85 per cent NaCl, and a butto of cells obtained. This button of cells was frozen and thawed two or three times. To the frozen and thawed cells was added 0.1 ml of normal rabbit serum as carrier. Approximately 10 μl of this mixture was withdrawn for autoradioimmunoelectrophoretic analysis. To the remaining mixture was added an additional 1.3 to 1.5 ml of carrier normal rabbit serum. After centrifugation, or centrifugation and Seitz filtration, this material was used for additional analysis including separation on sephadex G-200.

Gel Filtration on Sephadex G-200.—One ml of this mixture of cell extract and normal rabbit serum, to which 30 mg of sucrose had been added, was layered onto the surface of a 2.4 by 40 cm sephadex G-200 column. The column was equilibrated and eluted with 0.2 M NaCl, 0.2 M tris buffer, pH 8.0. Fractions were eluted in volumes between 3 and 5 ml, and the optical densities of each eluted fraction were recorded at 254 mμ by an LKB Model 4701 A unicord (Laboratorie och Kemikaliska Produkter Stockholm, Sweden). After separation of the carrier normal rabbit serum containing the radiolabelled cell proteins on sephadex G-200 the proteins contained in each eluted fraction were precipitated by addition of 1/4th volume of 20 per cent TCA, and each precipitate was washed once with 5 per cent TCA. The precipitated protein was then dried and dissolved in 1.0 ml hyamine. The hyamine-protein solution in each tube was transferred to a counting bottle, and each tube washed with a total of 10 ml of phosphore solution. The washings were added to the counting bottles and the amount of radioactivity in each was determined as described above.

Autoradioimmunoelectrophoresis.—Immunoelectrophoresis was carried out by a modification of the method of Scheidegger (10). After 48 hours, when the precipitate arcs were fully formed, the plates were washed in a stirred bath of 0.14 M NaCl which was changed several times during a period of 2 days. After a final brief wash in distilled water the plates were dried, and the agar layer was placed in contact with X-ray film (kodirex, Kodak Ltd., London) for periods up to 4 weeks. The film was developed with Kodak D19B developer. After the detection of radioactivity the precipitin lines were stained with 0.15 per cent Ponceau S in 3 per cent trichloracetic acid. Autoradio-Ouchterlony analyses were carried out in a similar manner using double diffusion in agar plates. The latter method was used primarily in an attempt to demonstrate radiolabel in IgM precipitin arcs formed by the reaction of cell extract-serum protein mixtures with antisera specific for IgM allotype Ms1 (11) that was present in the IgM of the donor of the cells and in the IgM of the carrier serum.

RESULTS

Relationship Between Protein, RNA and DNA Synthesis, Blast Transformation, and Mitosis.—The amount of incorporation of C^{14}-lysine into protein and of P^{32} into RNA and DNA, and the per cent blast transformation in control

lymphocyte cultures (normal rabbit serum and normal sheep serum) and in cultures stimulated with PHA, SF, antiallotype (As4) serum, and sheep anti-WRS from 12 to 96 hours are plotted in Fig. 1. Both control and stimulated cultures had a similar rate of protein production during the first 24 hours. During the second 24 hours protein production dropped off markedly in the control cultures, while the production of protein was maintained in the stimulated cultures, until the third 24 hours. Little incorporation of P^{32} into RNA occurred until after 12 hours. At 24 hours there was a marked uptake of P^{32} into the RNA obtained from the stimulated cultures, and a significantly less but definite uptake in the control cultures. This observation on control and stimulated cultures was supported by grain counts of autoradiographs made from cultures receiving tritiated cytidine. Almost all lymphocytes in each culture had one to three grains at 12 hours. While the number of grains per cell increased slightly, if at all, in the 24 hours control cultures, there was a marked increase in the grain count in a proportion of the lymphocytes in the stimulated cultures at 24 hours. P^{32} incorporation into RNA continued to increase in the stimulated cultures during the second 24 hours of culture, but dropped off to background levels in the control cultures. RNA synthesis continued to increase in the 3rd day cultures of SF stimulated cells, but levelled off in the PHA and antiallotype-treated cultures and decreased in the anti-WRS–stimulated cultures, so that the amount of RNA synthesis was significantly greater in the SF stimulated cultures during the 3rd day. RNA synthesis dropped off in all stimulated cultures by 96 hours. Blast transformation followed closely the onset of RNA synthesis in the stimulated cultures at 24 hours and a marked increase in the percentage of blast cells occurred at 48 hours. The per cent of blast cells continued to increase until 72 hours in the SF-treated cultures while a drop off was noted in the PHA and antibody-treated cultures by that time. DNA synthesis did not start until 48 hours in the stimulated cultures and reached a maximum during the 3rd day except the anti-WRS stimulated–cultures in which maximum DNA synthesis was recorded at 48 hours. No blast transformation or significant DNA synthesis was observed in the control cultures. Mitoses were first seen in the 48 hour cultures receiving stimulants and never represented more than 2 per cent of the lymphocytes in any stimulated culture. PHA induced the greatest degree of blast transformation (80 per cent), with SF and anti-WRS intermediate (50 per cent) and antiallotype serum least (30 per cent). In contrast SF induced approximately twice as much RNA and DNA synthesis as PHA or antiallotype serum, which were approximately equal in the regard. The RNA/DNA ratio was essentially 2:1 with each stimulant. The basic plan of the experiment illustrated in Fig. 1 was repeated three times, each time using lymphocytes from a different rabbit with essentially identical results.

Characterisation by Gel Filtration on Sephadex G-200 Columns of the Proteins

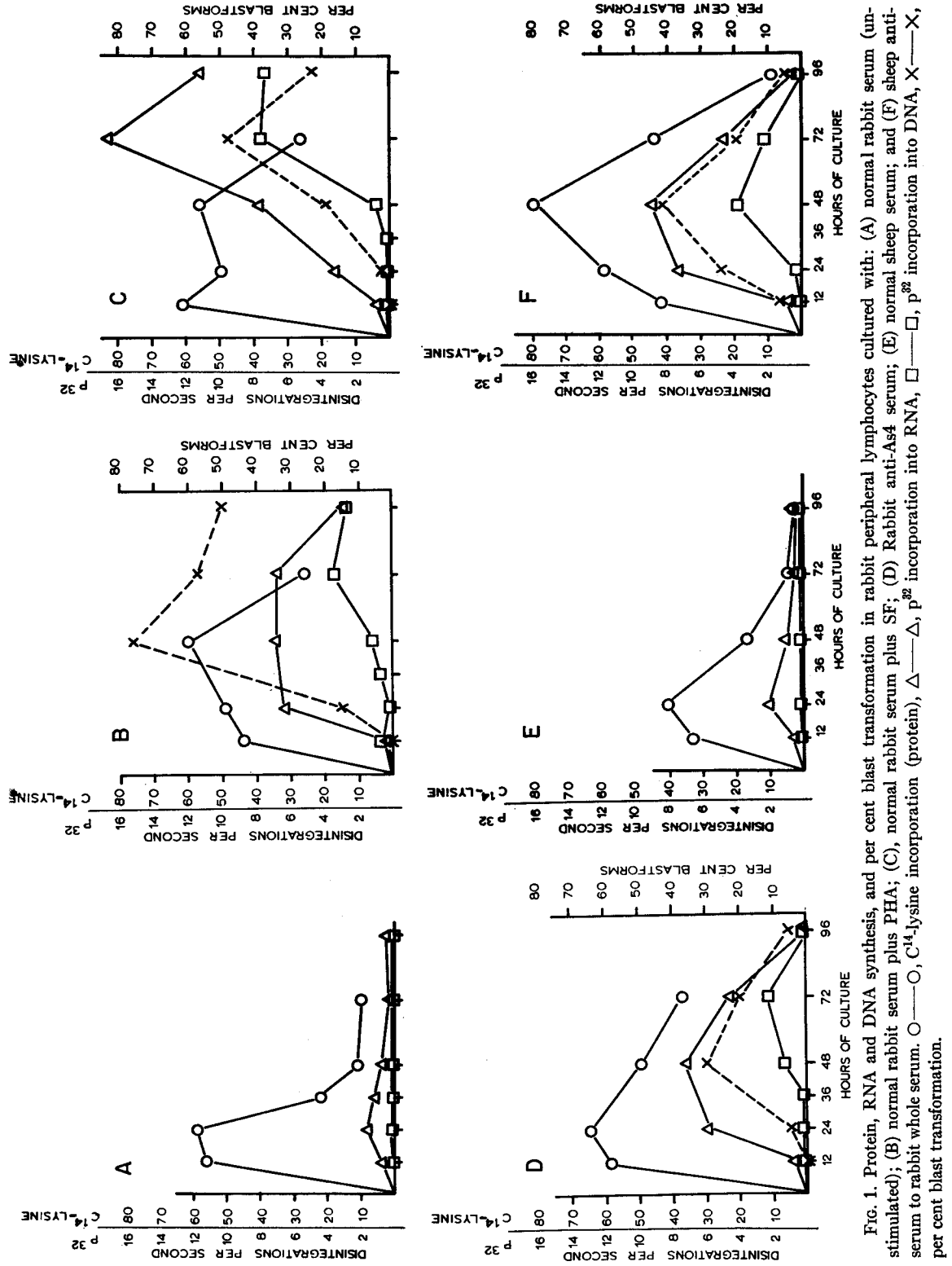


FIG. 1. Protein, RNA and DNA synthesis, and per cent blast transformation in rabbit peripheral lymphocytes cultured with: (A) normal rabbit serum (unstimulated); (B) normal rabbit serum plus PHA; (C), normal rabbit serum plus SF; (D) Rabbit anti-As4 serum; (E) normal sheep serum; and (F) sheep anti-serum to rabbit whole serum. ○——○, C^{14}-lysine incorporation (protein), △——△, P^{32} incorporation into RNA, □——□, P^{32} incorporation into DNA, ×——×, per cent blast transformation.

Synthesized by Lymphocyte Cultures.—Gel filtration on sephadex G-200 separates proteins according to their molecular size. Normal serum yields three peaks corresponding roughly to the 19S, 7S, and 4.5S peaks revealed by the ultracentrifuge. Prior to gel filtration rabbit serum was added to extracts of the cell cultures as described in the Materials and Methods section. The radio-

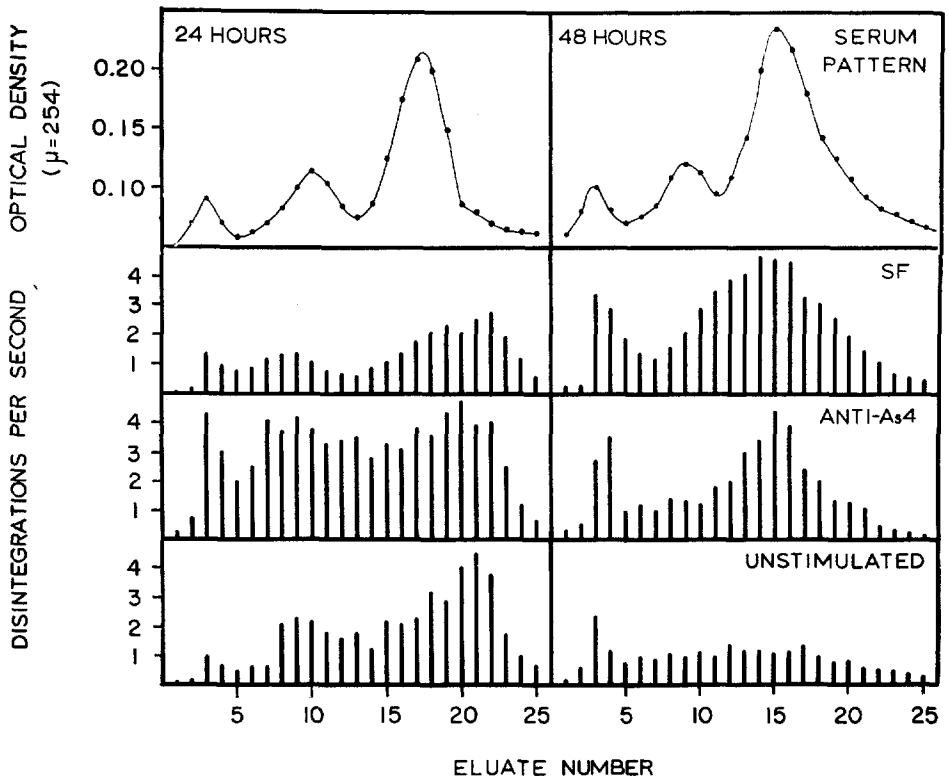


FIG. 2. Characterization of C^{14}-lysine-labelled cell products at 24 and 48 hours on sephadex G-200 in relation to carrier normal rabbit serum proteins; normal rabbit serum (unstimulated), anti-As4 serum, and staphylococcal filtrate (SF)-treated cultures.

activity of the column fractions obtained from the sephadex G-200 separation of the cell-extract–serum mixtures was due to the presence of C^{14}-lysine incorporated into protein by the cell cultures. Comparison of the distribution of radioactivity with the three main serum protein peaks provided an index of the molecular size of the proteins synthesized *in vitro* (Figs. 2 and 3). Fig. 2 depicts the pattern of radioactivity produced during the first and second 24 hours by unstimulated (control), by antiallotype stimulated, and by staphylococcal filtrate stimulated lymphocyte cultures. Fig. 3 shows the pattern of radioactivity at 24 and 48 hours produced by cultures stimulated by PHA and anti-

WRS serum. The cell-extract–serum protein mixtures producing the patterns of Fig. 3 were Seitz filtered before separation; the mixtures producing the patterns of Fig. 2 were not filtered. Other experiments demonstrated that prelimi-

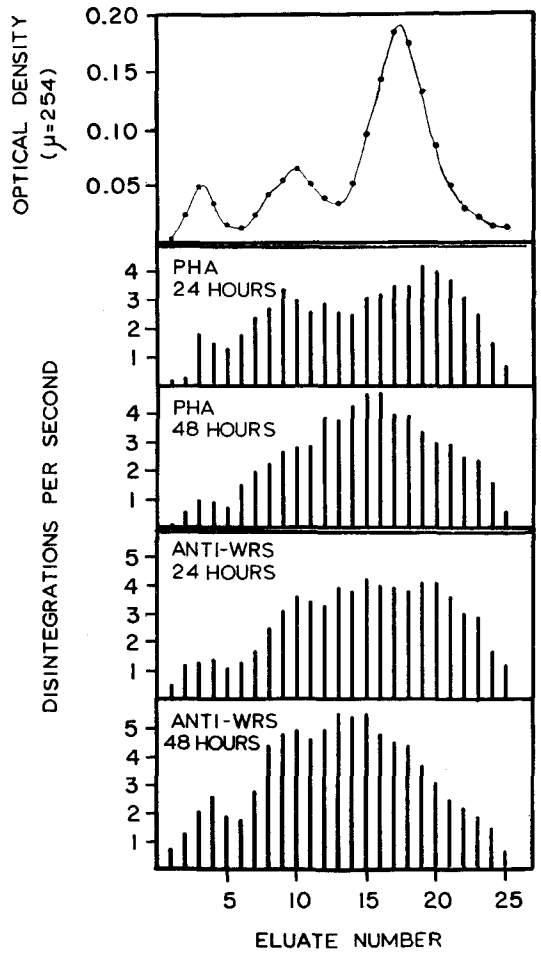


FIG. 3. Characterization of C^{14}-lysine–labelled cell products at 24 and 48 hours on sephadex G-200 in relation to carrier normal rabbit serum proteins: phytohaemagglutinin (PHA), and anti-WRS stimulated cultures.

nary Seitz filtration considerably reduced the radioactivity present in sephadex eluates corresponding to the region of the first serum protein peak, indicating that the major part of the radioactivity of this peak is associated with particles larger than serum proteins, most likely microparticulate matter from disrupted cells.

Analysis of Figs. 2 and 3 revealed the following characteristics of the synthe-

sized proteins. During the first 24 hours of culture, unstimulated and stimulated lymphocytes produced protein having a considerable range of molecular sizes, and the distribution within this range was similar for all cultures. A large amount of radioactivity was eluted after the third serum protein peak, indicating synthesis of proteins of smaller dimensions than serum albumin. A peak of radioactivity also appeared just before the second serum protein peak, presumably due to proteins of larger size than serum IgG (7S γ-globulins). During the second 24 hours of culture the pattern of synthesis changed. Synthesis by control cultures was very low but synthesis by stimulated cultures was maintained. The stimulated cultures continued to produce radiolabelled protein in a considerable range of sizes. The predominant peak now appeared before the third serum protein peak. This suggests that the majority of proteins synthesized was of a molecular size similar to or slightly greater than that of serum albumin. The anti-IgG and antiallotype stimulated cultures also produced a small peak of radioactivity that corresponded to the second serum protein peak, indicating the synthesis of proteins of a size similar to IgG (7S).

These findings show that a variety of stimulants have little effect in changing the overall pattern of protein synthesis during the first 24 hours of culture. The changes observed during the second 24 hours are very similar in all of the stimulated cultures. IgG cannot form a major proportion of the proteins synthesized by the cells in any culture, although the synthesis of some IgG cannot be ruled out by this technique.

Characterisation of the Proteins Produced by Lymphocyte Cultures by Autoradioimmunoelectrophoresis and Autoradio-Ouchterlony Analysis.—In contrast to the sephadex fractionation studies, these techniques provide evidence of the synthesis of individual serum proteins. Analysis of serum proteins was made by the use of antisera that reacted with a range of serum proteins and by antisera specific for rabbit IgG and IgM. The major precipitin arc with demonstrable radiolabelling had the electrophoretic mobility of an α-globulin (Fig. 4). C^{14}-lysine was incorporated into the protein producing this arc between 24 and 48 hours by both unstimulated and stimulated cultures. However, there was a considerable difference in the degree of labelling, with the unstimulated cultures producing only a barely identifiable labelled line and a significant increase in the anti-As4-, SF-, and PHA-stimulated cultures in that order. Analysis of extracts from unstimulated cultures after the first 24 hours failed to convincingly demonstrate any labelled line, but PHA stimulated culture extracts gave labelling of a α-globulin line in the same position as that seen in the second 24 hour cultures.

In addition to the above α-globulin, PHA-stimulated culture extracts appeared to label two β-globulins during the second 24 hour culture period (Fig. 4). These labelled precipitin lines occurred in an area where considerable radioactivity remained fixed to the agar. However, these were regarded as indi-

cating specific C^{14}-lysine incorporation since other precipitin arcs in this region were not labelled. The finding of these labelled lines in the extracts from the PHA-stimulated cultures may not mean that these proteins are specifically stimulated by PHA. The failure to demonstrate labelling of these lines in extracts from other cultures may be due to technical difficulties, or these proteins

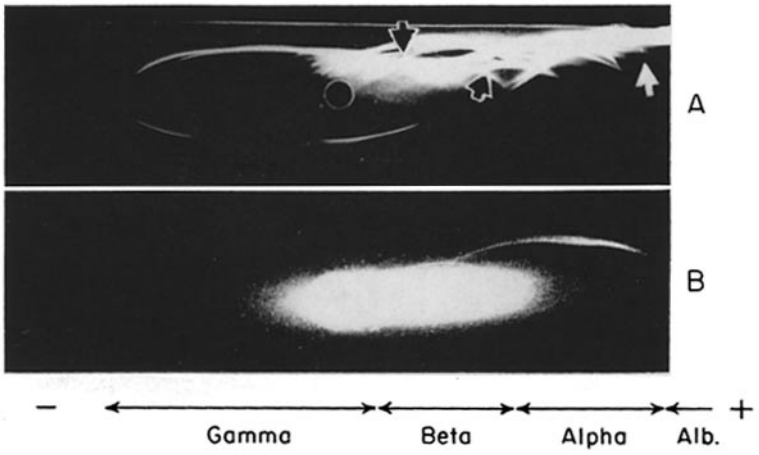


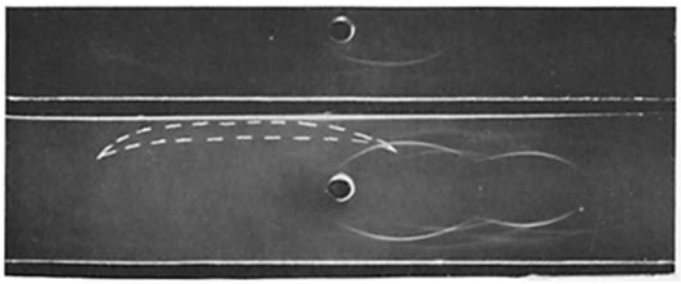
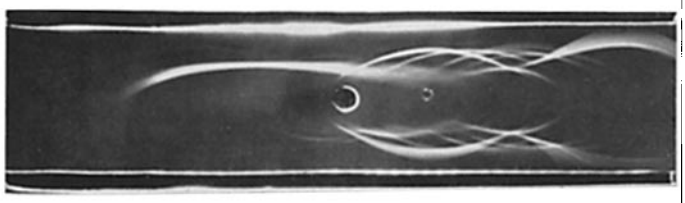
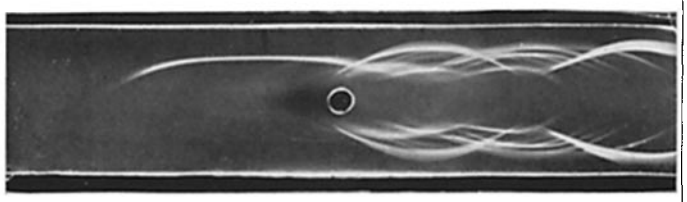
FIG. 4. Autoradioimmunoelectrophergram of C^{14}-lysine–labelled cell products from PHA-stimulated cultures. A is a photograph of the Ponceau S stained dried agar immunoelectrophoretic plate. Labelled cell extract-carrier serum mixture was placed in the center well and the proteins present separated by electrophoresis. A sheep antiserum to whole rabbit serum was then placed in the upper trough and a guinea pig antiserum specific for rabbit IgG in the lower trough. After development of the subsequent precipitin arcs for 48 hours, the slide was washed, the agar dried, and layered with X-ray film. After 4 weeks exposure, the film was developed and the labelled lines shown in B were revealed. The lines indicated by the arrows in "A" were indentified as those containing the radiolabell by overlying the stained agar plate with the developed film. A maximum of one α-globulin and two β-globulins were identified. The prominent IgG precipitin line did not contain demonstrable radiolabel in any preparation.

may not have been synthesized in an amount sufficient to be detected. The presence of radiolabel in the serum protein precipitin arcs due to binding of labelled enzymes on other materials and not *de novo* synthesis cannot be ruled out.

No evidence was found of synthesis of IgG using autoradioimmunoelectrophoresis. The autoradio-Ouchterlony test was primarily used to investigate labelling of IgM. Preciptin lines formed by reaction of the IgM allotype (Ms1) and specific anti-Ms1 serum were readily produced using double diffusion in agar. However, none of the precipitin lines formed contained detectable radio-

activity. Thus no direct evidence was obtained of any immunoglobulin synthesized by lymphocyte cultures *in vitro*.

FIG. 5. Effect of absorption by rabbit IgG on the transforming capacity of polyvalent sheep antisera to rabbit whole serum.

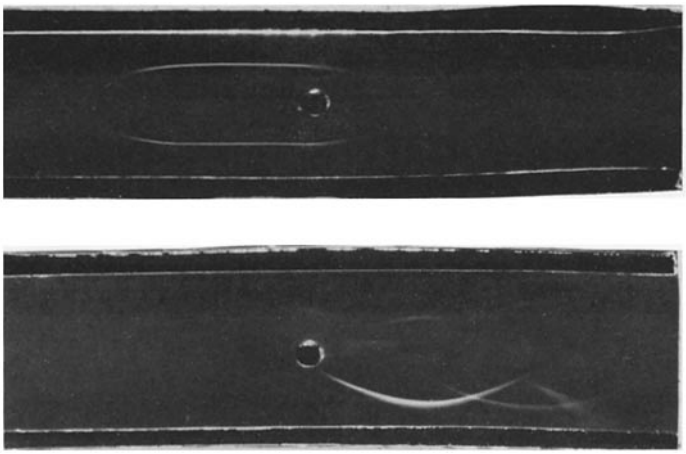
Immunoelectropherogram with NRS	Antiserum	Blast formation	C^{14}-thymidine uptake
		per cent	*counts/10 min.*
	368/I	14	1010
	368/I*	2	533
	368/V	39	6950
	368/V*	35	4960
	207/V	54	7640
	207/V*	45	6240

* Absorbed with rabbit IgG.

Transforming Capacity of Sheep Antisera to Rabbit Whole Serum after Absorption with Rabbit IgG.—Because specific anti-IgG sera had been shown to be effective in inducing blast transformation, it was first concluded that the transforming capacity of the anti-WRS must be due to the anti-IgG antibodies present in the polyvalent sheep antiserum to rabbit serum. However, it was

later decided that the transforming capacity might be due to antibodies to serum proteins other than IgG. In order to test this possibility three separate sheep antisera to rabbit serum protein were tested for transforming capacity with the same rabbit lymphocyte preparations before and after absorption with rabbit IgG (obtained by diethylaminoethyl-cellulose column fractionation of normal rabbit serum). The results of this experiment are presented in Fig. 5. Essentially complete removal of the precipitin line formed by the anti–IgG-IgG precipitin reaction was accomplished by prior absorption of the sheep antisera

FIG. 6. Effect of guinea pig antisera to rabbit serum proteins on rabbit lymphocyte cultures



Immunoelectropherogram with NRS	Anti-serum	Blast formation	C^{14}-Thymidine uptake
		per cent	*counts/10 min.*
	466	11	2060
	469	8	2190
	57	<1	274
	58	<1	178

with rabbit IgG. Although this absorption did significantly reduce the transforming capacity of one of the antisera tested (368/I), only a slight reduction occurred after absorption of the other two sera (207/V and 368/V). To determine if antisera to any rabbit serum protein might be effective inducers of blast transformation, two guinea pig antisera specific for rabbit IgG and two guinea pig antisera to rabbit serum proteins other than rabbit IgG were also tested and the results given in Fig. 6. Only the antisera to rabbit IgG induced blast transformation. The guinea pig antisera to rabbit serum proteins other than IgG produced relatively few and weaker precipitin arcs on immunoelectrophoresis with normal rabbit serum compared to the many arcs formed on the absorbed sheep antisera to rabbit serum.

DISCUSSION

The peripheral lymphocytes of rabbits stimulated *in vitro* with phytohaemagglutinin, staphylococcal filtrate, antiallotype serum, or heterologous antiserum to rabbit whole serum synthesize protein, RNA and DNA as indicated by the incorporation of radiolabelled precursor substances into these products. The synthesis of nucleic acids is almost entirely due to the activity of stimulated lymphocytes as there is only minimal labelling of the lymphocytes in the autoradiographs prepared from unstimulated cultures, and no cell types other than lymphocyte, lymphoblast, or intermediate form are labelled in any culture. Radioactive lysine is incorporated into protein by both the unstimulated and stimulated cultures during the first 24 hours, but during the second 24 hours incorporation only occurs in the stimulated cultures. All four stimulants provoke the same sequence of events; protein formation, followed after 24 hours by RNA synthesis and blast transformation, with DNA synthesis and mitoses by 48 hours. The present data on RNA and DNA synthesis are essentially the same as results previously reported for human lymphocytes cultured *in vitro* (4–8), except that a longer time period is required for stimulation of human lymphocytes.

Bach and Hirschhorn (2) demonstrated the incorporation of C^{14}-phenylalanine into protein produced by human lymphocytes stimulated *in vitro* with PHA. However, the control cultures of the above workers ceased to incorporate the radiolabelled phenylalanine into protein after only 2 hours, while the stimulated cultures continued to synthesize protein for 24 hours. This is in contrast to the present observations on rabbit lymphocytes which indicated no difference in the amount or type of protein synthesis in unstimulated and stimulated cultures until after 24 hours. The difference may be due to the different labelled amino acid used, to the different culture conditions, or to the different species of lymphocyte. It should be noted that the lymphocytes obtained from the peripheral blood of normal rabbits are morphologically much more uniform than those of human origin, and almost all the rabbit lymphocytes are morphologically similar to what would be termed "small lymphocytes" in man.

Characterization of the radiolabelled proteins synthesized by stimulated and non-stimulated rabbit lymphocytes *in vitro* failed to demonstrate the production of any immunoglubulins. Several authors have noted synthesis of IgG by *in vitro* lymphocyte cultures. The incorporation of labelled amino acid precursors into γ-globulin (IgG) by the peripheral white blood cells of rabbit (12), monkeys (13), and human (14) has been reported. However, the rabbits were hyperimmunized and plasma cells were seen in smears of the peripheral blood (12), the monkeys were infected with pneumococci (13) and the human cultures required over tenfold greater numbers of lymphocytes than the cultures used in the present study in order to demonstrate IgG synthesis and the preparations used contained 0.1 per cent plasma cells (14). Peripheral leucocytes obtained from

hyperimmunized animals may form specific antibody *in vitro* to the antigen with which the leucocyte donor was immunised (12, 15), and Elves *et al.* (3) claim that the peripheral lymphocytes from a sensitized human donor can be stimulated *in vitro* with antigen to synthesize specific antibody. There is some evidence that PHA may non-specifically stimulate an increase *in vitro* in the antibody content of lymph node fragment cultures from immunised animals (16). However, in none of these reports has the *in vitro* induction of IgG synthesis by non-specific stimulation of peripheral lymphocytes from a normal donor been demonstrated.

The observation that cultures of peripheral rabbit lymphocytes non-specifically stimulated to transform *in vitro* into blast forms do not synthesize measurable amounts of IgG does not agree with the interpretation of the data of Bach and Hirschhorn on human lymphocytes (2). The conclusions of these authors are based primarily on the results of specific cytoplasmic staining of transformed cells with fluorescein-conjugated antisera. As indicated by Robbins (17), the experimental details concerning the specificity of the antisera used by Bach and Hirschhorn has not been adequately described to permit satisfactory evaluation. Attempts in our laboratory to obtain fluorescent staining of transformed rabbit lymphocytes using antisera specific for rabbit IgG (18), and antisera specific for rabbit allotypes (19) have been consistently inconclusive and positive fluorescent staining of the cytoplasm of transformed lymphocytes has been obtained using fluorescent-conjugated antibody to whole rabbit serum or rabbit albumin (18). Bach and Hirschhorn (2) also claim that the electrophoretic migration of radiolabelled protein obtained from PHA stimulated human lymphocyte cultures was identical with serum γ-globulin. However, this result could be due to the cathodal migration of materials other than γ-globulin. The *in vitro* stimulation of immunoglobulin synthesis by non-specifically stimulated peripheral lymphocytes from normal donors cannot be ruled out completely. The finding that only a small fraction of the radiolabelled cell-extract is eluted from a sephadex G-200 column in the fractions where immunoglobulins would normally be found indicated that even if some immunoglobulins synthesis occurs it could not account for more than a very small proportion of the total protein made. The distribution of radiolabelled cell products obtained from human peripheral lymphocyte cultures on sephadex G-200 has led to a similar conclusion (19).

The broad distribution of radiolabelled material throughout the effluents of labelled cell-extract carrier serum mixtures emerging from the sephadex G-200 column shows that proteins with a wide range of molecular sizes are being synthesized by the lymphocyte cultures. The demonstration by autoradioimmunoelectrophoresis of labelling in a maximum of only three serum proteins (non-immunoglobulins) suggests that the majority of proteins produced by the cells *in vitro* are not serum proteins. Indeed, attempts to precipitate radioactivity

from labelled cell extract-carrier serum mixtures with antisera to rabbit IgG were unsuccessful, and only a small fraction of the radioactivity precipitable with trichloracetic acid could be precipitated with antisera to whole rabbit serum.

The proteins produced by peripheral lymphocytes stimulated *in vitro* are most likely cellular proteins related to the metabolic alterations necessary for mitosis. Using time lapse phase contrast cinemicrography Marshall *et al.* (20) were able to observe the first mitosis of PHA-stimulated human lymphocytes *in vitro* after 67 hours. The daughter cells formed subsequently were seen to divide themselves after an additional 23 hours. In a previous report (1) we indicated that the histologic end point of blast transformation appeared ultimately to be mitosis and not differentiation into plasma cells.

The observation that sheep antisera to rabbit whole serum induce significant blast transformation in cultures of rabbit lymphocytes even after absorption of the sheep antisera with rabbit IgG indicates that the induction of transformation is not dependant on the IgG system alone; *i.e.*, antibodies to serum proteins other than IgG must also be capable of inducing blast transformation. Other experiments have indicated that the allotypic specificity of rabbit lymphocytes as brought out by blast transformation is not alterable by environmental IgG and therefore must be due to IgG manufactured in the cells (1, 23). One possible explanation for the finding that heterologous antibodies other than anti-IgG are able to induce blast transformation is that the small lymphocytes of the rabbit manufacture certain other serum proteins in addition to IgG and that blast transformation can be induced with antibodies to these other proteins. However, as the sheep antisera used in the present study were obtained after immunisation with whole rabbit serum, antibodies to cellular materials that may have been present as contaminants in the rabbit serum used as the original antigen cannot yet be ruled out as being the effectors of blast transformation. The results with guinea pig antisera show; (*a*) that specific anti-IgG may induce blast transformation, and (*b*) that antisera to other serum proteins do not always induce blast transformation.

The main finding supporting a specific immunologic reaction as the initiating mechanism in transformation has been the ability of specific antigen to induce transformation in cultures of human lymphocytes from sensitised donors (17). The only differences yet recorded between antigen-specific blast transformation of lymphocytes from sensitised donors and non-specific blast transformation (*i.e.* PHA, SF) has been the smaller proportion of cells transformed with antigen (up to 20 per cent) compared to PHA (up to 100 per cent) and a somewhat longer time required for stimulation with antigen (17). As yet, antigen-specific transformation has not been observed in peripheral lymphocyte cultures obtained from immunised rabbits and guinea pigs (18). In addition to the blastogenic effect of anti-IgG and antiallotype sera on rabbit lymphocytes

in vitro, it has been shown that rabbit antisera to human leucocytes are also powerful inducers of lymphoblast transformation *in vitro* (21). It is not unlikely that the effectiveness of these rabbit antisera may be due to antibody against human serum protein. As yet no histologic or essential metabolic difference between blast cells formed after stimulation with any of the different agents has been reported. Therefore, the net effect of the different agents on lymphocytes appears to be the same; *i.e.*, the induction of metabolic changes culminating in mitoses. However, there is not yet enough information available to judge whether the initiating event is similar or different with different stimulants. Differences in the recognition of antigen as a specific stimulator and PHA as a non-specific stimulator have been postulated (22).

A common mechanism for the specific and the non-specific induction of lymphocyte transformation may be the ability of the given stimulant to react with protein present in, or manufactured by, the lymphocyte. Thus, of the agents that induce blast transformation, antisera to allotypic determinants, to IgG, or to other serum proteins react specifically with serum protein; antileucocyte serum may well stimulate by a reaction with serum proteins; PHA and other bean seed extracts react with serum proteins to form precipitates (24, 25); and one of the properties of antigen is to react with antibody, either serum antibody or antibody present in the cell. Reactions between SF and serum proteins have not been reported, and attempts in our laboratory to demonstrate such a a reaction using precipitin and double diffusion in agar analyses have been unsuccessful (19). However, SF may act directly on a cellular protein (or proteins) not present in serum.

SUMMARY

In vitro cultures of the peripheral blood lymphocytes of rabbits may be stimulated with phytohaemagglutinin, staphylococcal filtrate, antiallotype serum, or sheep anti-rabbit whole serum to synthesize protein, RNA and DNA as indicated by the incorporation of radiolabelled precursor substances into these products. A sequence of events found in all stimulated cultures characteristically shows protein synthesis followed by RNA synthesis, histologic blast transformation, DNA synthesis, and mitosis, with the complete sequence requiring 48 hours. All four stimulants induce essentially identical metabolic changes. Characterization of the proteins synthesized by lymphocytes *in vitro* has failed to demonstrate immunoglobulin synthesis by stimulated or non-stimulated cultures. It is concluded that the majority of proteins produced by peripheral lymphocytes stimulated *in vitro* are most likely cellular proteins related to the metabolic alterations necessary for mitosis.

Absorption of sheep antisera to whole rabbit serum with rabbit IgG does not always remove the transforming capacity of the sheep antisera. Thus, it is likely that antibodies to proteins other than IgG present in the small lymphocyte may

also be able to stimulate transformation. A possible common mechanism for the induction of lymphoblast transformation may be the ability of both specific and non-specific stimulants to react with protein constituents of the lymphocyte which may also be present in serum.

The authors would like to thank Miss Jennifer Lowe for her excellent technical assistance.

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