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Interaction between 'Sensitized Lymphocytes' and Antigen *In Vitro*

I. THE RELEASE OF A SKIN REACTIVE FACTOR

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Summary. Peritoneal exudate lymphocytes (PEL), purified on glass beads and lymph node (LN) cells from guinea-pigs immunized with tubercle bacilli were cultured for 24 hours, in serum-free medium, without and with various concentrations of Tuberculin PPD.

Supernatants obtained from cultures with 10 μg PPD/10^7 lymphocytes provoked an intense inflammatory reaction, when injected into the skin of normal guinea-pigs. PEL were more active than LN cells from the same animals. The reaction was characterized by erythema and induration, with a peak between 3 and 6 hours and histologically a mixed polymorphonuclear–mononuclear infiltrate in the dermis was seen. When fractionated on Sephadex G-200, skin activity of both PEL and LN supernatants was concentrated in a peak corresponding to the molecular weight of serum albumin, while in LN material some activity was also present in a small molecular weight peak. The active material could be separated from albumin by chromatography on DEAE-cellulose. Immunoelectrophoretic analysis of skin reactive peaks detected a slow α-globulin in both PEL and LN supernatants. PPD in the form of a complex with a protein precipitated by anti-γG antiserum, was detected in the skin-active Sephadex peak III of LN supernatants, by radio-immunoelectrophoresis. Skin activity was precipitated with ammonium sulphate at 66 per cent saturation and was destroyed by pepsin treatment. Formation of the skin-active material was depressed by Puromycin and Actinomycin-D and the development of skin inflammation was suppressed by pretreatment of the recipient with anti-lymph node extract serum.

Evidence for antigen induced specific synthesis and release of an α-globulin in PEL and LN cultures was found but its relation to the skin active material is unknown.

INTRODUCTION

It is assumed that the efferent arc of the immune response in delayed hypersensitivity is initiated by a direct interaction of a small number of sensitized cells and specific antigen. The inflammatory response which follows is made up by a majority of non-committed cells, the arrival and settling of which is mediated through an unknown mechanism. One way to understand this process is by using simple *in vitro* systems in which lymphoid cells from immunized animals are incubated with antigen. Such exposure to antigen was found

to result in mitosis (Pearmain, Lycette and Fitzgerald, 1963), cell damage (O'Neill and Favour, 1955) and inhibition of macrophage migration (Rich and Lewis, 1932; David, Al-Askari, Lawrence and Thomas, 1964).

Very recently it has been found that supernatants of immune lymphocytes cultured in the presence of antigen, possess an inflammatory activity, when injected into normal skin (Bennett and Bloom, 1968; Dumonde, Howson and Wolstencroft, 1968).

The purpose of the experiments to be described, was to define the conditions necessary for the formation of inflammatory factors in a well defined lymphocyte–antigen system, to characterize the active material and to determine the mechanism of its action. It is suggested that formation of similar soluble substances, at the skin test site, *in vivo*, might take part in the initiation of the typical inflammation of delayed hypersensitivity reactions.

MATERIALS AND METHODS

Experimental animals

Male Hartley guinea-pigs, weighing more than 500 g were used both as cell donors and as test animals.

Immunization

The guinea-pigs were immunized with Freund's complete adjuvant (CFA, Difco, containing killed *Mycobacterium tuberculosis* H_{37} Ra, 0·5 mg/ml) in a dose of 1 ml/animal, 0·6 ml divided among the four footpads and 0·4 ml given subcutaneously into the nuchal region. Four weeks later all animals responded with typical delayed type reactions to the intradermal injection of 2 μg Tuberculin PPD (Mammalian PPD—Ministry of Agriculture's Central Veterinary Laboratory, Weybridge) and lymphocytes were uniformly obtained at this time.

Animals were immunized in groups of ten or twenty guinea-pigs, and lymphocytes were harvested 4 weeks later, pooled and cultured in one experiment.

Antigen

Tuberculin PPD obtained as a 2 mg/ml solution, containing glycerine and phenol, was used throughout this work. For both skin testing and *in vitro* experiments, the above material was dialysed for 48 hours against several changes of phosphate buffered saline, pH 7·2 (PBS) at 4° and kept frozen at −20° in 2-ml aliquots.

Cell harvest

Two types of lymphoid cells were used in all experiments, from the draining lymph nodes and from oil induced peritoneal exudates. Lymph nodes draining the four footpads were minced with scissors, under sterile conditions, in ice-cold supplemented Eagle's Minimal Essential Medium (Supp. Eagle's MEM, Burroughs Wellcome and Flow Laboratories) and free cells separated from tissue fragments by filtering through a 100 mesh sterile nylon gauze. The cells were washed twice in Supp. Eagle's MEM by centrifuging at 325 ***g*** for 15 minutes at 4°. The washed cells were resuspended in the final culture medium as described later on. The proportion of living cells was determined at this stage by the trypan blue method (Ballantyne and Stetson, 1964) as approximately 50 per cent.

The same guinea-pigs had been injected, intraperitoneally, 4 days before cell harvest with 30 ml sterile light paraffin oil (British Drug Houses). Exudates were removed by

washing the opened peritoneal cavity, under sterile conditions, with 150 ml Earle's Balanced Salt Solution (Earle's BSS, Burroughs Wellcome) and filtered through a double layer of 100 mesh nylon gauze into ice-cooled conical flasks. The cells were washed three times in Earle's BSS at 4°, which process successfully removed the paraffin oil present in the exudates and resuspended in Supp. Eagle's MEM containing 20 per cent heat inactivated foetal calf serum (Flow Laboratories) to a concentration of $5–10 \times 10^7$ cells/ml. The relative proportion of lymphocytes in these exudates varied between 25 and 35 per cent.

Separation of lymphocytes and macrophages

In order to obtain a pure lymphocyte suspension, exudate cells were fractionated as described by Rabinowitz (1964) and Plotz and Talal (1967). Glass beads 60 mesh, (British Drug Houses) were washed in concentrated HCl for 15 minutes and profusely rinsed with tap-water followed by distilled water. The dried beads were packed into 50-ml Pyrex separating funnels (11 cm long, internal diameter 25 mm) and supported by a glass wool plug. The funnels were filled with serum containing medium warmed at 37° and incubated for 15 minutes at 37°. Ten millilitres of exudate cell suspension ($5–10 \times 10^8$ cells) were added to one funnel and macrophage adherence allowed to proceed for 20 minutes at 37°. At the end of this interval lymphocytes were eluted with 30 ml of warm serum containing medium at a speed of 1·5 ml/min. The lymphocyte suspension was spun at 325 *g*, washed once with serum-free Supp. Eagle's MEM and resuspended in the final culture medium. By this procedure the percentage of lymphocytes was increased to 95–97 per cent. The proportion of living cells was constantly higher than 95 per cent.

In order to obtain purified macrophages the peritoneal exudate cells were incubated in horizontally placed 250-ml 'Falcon' tissue culture flasks (Falcon Plastics) at a concentration of 5×10^6 cells/ml in Eagle's medium containing 20 per cent normal guinea-pig serum. After repeated removal of unattached lymphocytes the macrophages were released from the flask surface by exposure to EDTA as described by Bloom and Bennett (1966), centrifuged and washed once in medium.

Culture medium

The basis for the culture medium used for incubating both lymph node (LN) and peritoneal exudate lymphocytes (PEL) in the presence of antigen, was Eagle's MEM which was supplemented with non-essential amino acids; sodium pyruvate, 0·001 M; L-glutamine, 0·002 M; Penicillin 100 u/ml and Streptomycin 100 μg/ml. A number of experiments were done in the above medium additioned with 20 per cent heat inactivated normal guinea-pig serum, especially when attachment of macrophages to the culture flask surface was a desired phenomenon. However, the vast majority of cultures described in this paper were performed in a serum-free medium containing Supp. Eagle's MEM and 0·2 per cent Carbowax 20M (Pospíšil, 1967) as a serum replacer (Carbowax medium). Carbowax 20M (Union Carbide) is a polyethylene glycol with a molecular weight of 15,000 and was chosen after it was found that the percentage of living cells was higher than that seen after culture in Supp. Eagle's MEM without any serum replacer or in the presence of Polyvinyl pyrrolidone (0·1 per cent), methyl cellulose (0·12 per cent) and Dextran (molecular weight = 110,000, 1·2 per cent). Additional reasons for the choice of Carbowax 20M were the facts that it had no inflammatory properties when tested as a 0·2 per cent solution in normal skin and that its molecular weight allowed easy localization during gel filtration.

Tissue culture

LN cells were suspended in Carbowax Medium at a concentration of 5×10^7 cells/ml, PEL at 1×10^7 cells/ml.

Whole peritoneal exudate cells were cultured at a concentration of 2×10^7 cells/ml, while separated macrophages were cultured at a concentration of 5×10^6 cells/ml. Five millilitres of the cell suspensions were pipetted into 30 ml 'Falcon' sterile, disposable tissue culture flasks. PPD was added in two concentrations: 0·2 and 10 μg/10^7 cells, in a volume of 0·1 ml/flask and control cultures were left without addition of antigen. The flasks were placed in a horizontal position with loose caps in special airtight lucite boxes which were gassed with 90 per cent air–10 per cent CO_2 and then hermetically closed and placed in a 37° incubator for 8–24 hours.

At the end of the culture interval suspensions were centrifuged at 1000 ***g*** for 30 minutes at 4°. Supernatants were removed and clarified by recentrifugation at 25,000 ***g*** for 1 hour. All sediments were resuspended in PBS, using 1 ml of buffered saline for 3 ml of suspension and sonicated in a 60 W Ultrasonic Disintegrator (MSE) at 20 kcycles/sec for 3 minutes in a ice-cooled test tube. Sonicates were also freed of insoluble material by centrifugation at 25,000 ***g*** for 1 hour. Such supernatants are subsequently referred to as cell extracts.

Skin testing

Culture supernatants and cell extracts from cultures incubated with or without antigen were tested for skin activity, without any previous concentration, by intradermal injection of 0·1 ml into the skin of normal, non-immunized guinea-pigs. Supernatants of cultures without antigen addition were supplemented with PPD 50 μg/ml, added just before injection (antigen supplemented samples). Up to six skin tests were simultaneously performed on a single guinea-pig, one of them invariably consisting of a control injection of 5 μg PPD. Supernatants, extracts and fractionation products were always tested in duplicate, at least. Reactions were assessed at 3, 6 and 24 hours after testing according to the following four criteria (Turk and Polák, 1968): (a) degree of erythema, expressed by a numerical value as follows: red (3), pink (2), pale pink (1), faint pink (0·5) and negative (0); (b) diameter of erythema in millimetres; (c) induration, which was expressed as the double thickness of the skin at the site of injection minus the mean double thickness of skin on either side of the reaction, as measured by using the Schnelltäster (System Kröplin, Type A. 02 T, H. C. Kröplin, Schlüchtern, Hessen, Germany); and (d) absence or presence of haemorrhage. Skin activity was considered to be present when a clear cut difference was apparent, as far as one or more of the above criteria was concerned, between antigen incubated and antigen supplemented supernatants or extracts.

Histology

Biopsies were taken from the intradermal injection site at 6 and 24 hours, fixed in corrosive acetic acid fixative, and stained with haematoxylin and eosin.

Effect of inhibitors

Puromycin dihydrochloride (Nutritional Biochemicals Corporation, Cleveland, Ohio), Actinomycin D (Merck Sharp and Dohme, Rahway, N. J.) and Chloramphenicol (Carlo Erba, Milano) were added to the culture medium in order to assess their effect on the formation of skin active material. ε-Aminocaproic acid (Hopkin and Williams) and Soya bean trypsin inhibitor (Sigma Chemical Company) were also used with the same purpose.

Immunoelectrophoresis (IEP)

Culture supernatants and cell extracts were examined by micro-immunoelectrophoresis (Scheidegger, 1955) on 3×1 in. microscope slides covered with 1 per cent agar ('Ionagar', Oxoid Ltd), using barbitone buffer, pH: 8·2, ionic strength 0·04 in both the gel and electrode compartment. Electrophoresis was performed by applying 5 V/cm for 1 hour. To the troughs a battery of three antisera was applied: rabbit anti-guinea-pig whole serum (AGPS), anti-guinea-pig lymphocyte (ALS) and anti-guinea-pig lymph node extract (ALNEx) and diffusion allowed to proceed for 48 hours at 4°. The slides were washed for 48 hours, dried and stained with 0·2 per cent Amido Black 10B. On special occasions precipitin lines were stained for glycoprotein, lipid, nucleoprotein and peroxidase as described by Uriel (1964).

Production of antisera

Anti-whole guinea-pig serum (AGPS) was produced by injecting rabbits twice at a 1-week interval with 2 ml of a 1 : 1 emulsion of guinea-pig serum in Freund's incomplete adjuvant (Difco) given in the footpads and subcutaneously and followed by one subcutaneous injection 1 week later. The animals were bled 7–14 days from the last injection.

In order to obtain anti-lymphocyte serum (ALS) rabbits were injected intravenously with $7{\cdot}5\times10^8$ fresh guinea-pig lymph node cells (cervical and mesenteric) which were washed twice in Earle's BSS. Two weeks later the rabbits were boostered intravenously with $1{\cdot}5\times10^9$ lymph node cells and bled out 1 week after the second injection. Antiserum against cell-free lymph node extract (ALNEx) was prepared in New Zealand White rabbits following the procedure of Willoughby, Walters and Spector (1965).

Incorporation of ^{14}C-labelled amino acids

LN cells and PEL were cultured for 24 hours in Eagle's MEM deficient in leucine (Burroughs Wellcome) containing 0·5 per cent ovalbumin (crystallized, salt-free, Sigma Chemical Company), to which ^{14}C-labelled L-leucine ([^{14}C]L-leucine) (specific activity 6·5–10 mC/mM. The Radiochemical Centre, Amersham) was added at a concentration of 1 μC/ml (Van Furth, Schuit and Hijmans, 1966). The cell density was 2×10^7 cells/ml for LN and 1×10^7 cells/ml for PEL and PPD was added in two concentrations: 0·2 and 10 μg/10^7 cells, some flasks being incubated without addition of antigen. In one experiment Puromycin at a concentration of 50 μg/ml was added to some flasks while parallel cultures of the same cells were incubated without the antibiotic. After 24 hours in culture, supernatants and cell extracts were prepared as usual and dialysed for 72 hours, at 4°, against six changes of PBS containing L-leucine 0·5 mg/ml, Penicillin 100 U/ml and Streptomycin 100 μg/ml. Samples were then concentrated by vacuum dialysis in collodion bags (Sartorius Membranfilter, Göttingen) to one-tenth of the original volume and submitted to immunoelectrophoresis by adding the concentrated culture products three times to the antigen well. The same samples were also run after first filling the well with Freund's complete adjuvant immunized guinea-pig serum, acting as carrier. After precipitation with AGPS, ALS and ALNEx the immunoelectrophoretic slides were washed for 72 hours at 4° with three changes of PBS per day, dried and placed into close contact with 'Kodak' Panchro Royal film, in light tight boxes for an exposure of 60 days. The films were developed for 25 minutes in 1 : 1 dilution of 'Promicrol' developer (May & Baker) and fixed in 'Unifix' (Kodak).

Labelling of PPD with ^{125}I

Tuberculin PPD was trace-labelled with ^{125}I (sodium iodide, free from reducing agent, The Radiochemical Centre, Amersham) by the method of McConahey and Dixon (1966). Non-protein bound iodide was removed by dialysis in the cold for 48 hours against 0·1 M phosphate buffer, pH 7. Specific activities varied between 0·29 and 0·57 $\mu c/\mu g$ of PPD. All isotope counting operations were performed in a well-type scintillation counter (Ekco Electronics).

[^{125}I]PPD radio-immunoelectrophoresis

Culture supernatants and cell extracts were examined for binding of PPD by radio-immunoelectrophoresis as described by Yagi, Maier, Pressman, Arbesman and Reisman (1963). Industrial G type X-ray film (Ilford) was used for exposure to the dried slides for 2–14 days, after which time interval the films were developed for 6 minutes with 'Phen-X' developer (Ilford) and fixed with 'Unifix' (Kodak).

Gel filtration and chromatography

Active supernatants were freeze-dried, redissolved in one-tenth of the original volume of distilled water and dialysed against successive changes of distilled water and PBS in the cold. The concentrated equivalent of 50–225 ml supernatant was applied on $100 \times 2{\cdot}5$ cm columns of Sephadex G-200 (Pharmacia, Uppsala) pre-swollen in PBS. All columns were eluted with the same buffer at 4°, at a flow rate of 9 ml/hr and 3-ml fractions were collected. Optical densities were determined at three wavelengths: 280, 260 and 215 mμ (Bennett and Bloom, 1968).

In some experiments [^{125}I]PPD was used in culture and radioactive antigen containing supernatants were fractionated on Sephadex G-200. In this case fractions were read for optical density and additionally counted for radioactivity, in order to determine the localization of antigen in the eluted fractions.

Peaks obtained by plotting the optical densities were concentrated by freeze-drying followed by redissolving in one-tenth of the volume of the sample applied on the column and brought to isotonicity by dialysis against PBS in the cold. This was followed by centrifugation at 25,000 *g* for 1 hour to remove insoluble material and the clarified peaks were tested for skin activity and by immunoelectrophoresis as indicated above.

Active Sephadex peaks were further fractionated by ion-exchange chromatography on DEAE-cellulose (Whatman). Columns, $45 \times 1{\cdot}5$ cm, of the anion-exchanger were equilibrated with 0·01 M potassium phosphate buffer, pH 8, and 5-ml fractions were eluted by a succession of 0·01 M and a 0·01–0·3 M potassium phosphate buffer gradient, pH 8 (Sela, Givol and Mozes, 1963). Protein peaks were concentrated as indicated before and tested for activity in the skin and by immunoelectrophoresis.

Incubation with phytohaemagglutinin (PHA)

CFA immunized guinea-pig LN cells and PEL were cultured for 24 hours in the presence of PHA-P (Difco) without addition of PPD. PHA-P was reconstituted with 5 ml of distilled water and from this solution a 1 : 30 dilution was made in Carbowax medium. Volumes of 0·02, 0·04 and 0·06 ml of the 1 : 30 dilution of PHA-P were added per millilitre of cell suspension containing 5×10^7 lymph node cells and 1×10^7 peritoneal exudate lymphocytes, respectively. Culture supernatants were tested for activity in normal skin.

Enzyme treatment of culture supernatants

Trypsin. Two-millilitre volumes of culture supernatant were dialysed against 0·1 M potassium phosphate buffer, pH 7·6, and digested with insoluble trypsin (Enzite-Try, Seravac Laboratories) 10 mg/ml for 6 hours at 37°, with continuous shaking. The digested samples were centrifuged at 1400 ***g*** to remove the enzyme.

Pepsin. Two millilitres of supernatant were dialysed against 0·1 M acetate buffer, pH 4·1, and digested with pepsin (twice crystallized, 2650 U/mg, Sigma Chemical Company) 250 μg/ml for 16 hours at 37°.

Papain. Two-millilitre samples, dialysed against 0·1 M potassium phosphate buffer, pH 7·0, containing 0·002 M sodium EDTA and 0·01 M cysteine, were digested with insoluble papain (Enzite-Pap, Seravac Laboratories) 10 mg/ml for 16 hours at 37° with continuous agitation. Digests were centrifuged at 1400 ***g*** in order to obtain enzyme-free material.

Ribonuclease. Two and a half millilitres of supernatant were dialysed against 0·1 M potassium phosphate buffer, pH 7·6, and incubated for 1 hour at 37° with 60 μg/ml of bovine pancreas ribonuclease (five times crystallized, protease free, Sigma Chemical Company) previously heated for 15 minutes at 60°.

Deoxyribonuclease. Two and a half millilitres of culture supernatant dialysed against 0·1 M potassium phosphate buffer, pH 6·5, containing 0·016 M magnesium sulphate were incubated with 60 μg/ml of bovine pancreas deoxyribonuclease (once crystallized, Sigma Chemical Company) for 1 hour at 37°.

All enzyme digests were dialysed overnight against PBS at 4°, centrifuged at 25,000 ***g*** for 30 minutes and tested in normal skin. For each particular enzyme three controls were included in the experiment: one culture sample dialysed against the enzyme buffer and incubated for the same length of time at 37° but without enzyme, a second sample treated as the first but kept at 4° and a third control containing buffer and enzyme but no culture supernatant and incubated at 37°.

For insoluble enzyme experiments, controls one and two contained 10 mg/ml of Carboxymethyl-cellulose, which is the insoluble matrix to which the enzymes are linked.

Ammonium sulphate precipitation

A saturated solution of ammonium sulphate, brought to pH 7·0 with ammonium hydroxide was added to 3-ml aliquots of skin active supernatant, in quantities meant to give final concentrations of 40, 46, 53, 60 and 66 per cent saturation. The mixtures were incubated overnight at 4° and centrifuged at 75,000 ***g***. The precipitates were redissolved in 2 ml distilled water and both supernatants and sediments were freed of ammonium sulphate by dialysis against distilled water followed by PBS in the cold. All samples were concentrated by vacuum dialysis to the same volume of 0·5 ml, centrifuged at 25,000 ***g*** and tested in normal skin.

RESULTS

SKIN ACTIVITY OF WHOLE CULTURE SUPERNATANTS

Injection of supernatants of sensitized LN and PRL, cultured in the presence of PPD into normal guinea-pig skin, induced a pronounced inflammatory reaction characterized by erythema and induration. Both parameters reach their maximum 3–6 hours after skin

TABLE 1

SKIN INFLAMMATORY REACTIONS INDUCED BY SUPERNATANTS OF SENSITIZED MONONUCLEAR CELLS, INCUBATED WITH SPECIFIC ANTIGEN

Type of cells in culture from which supernatants originate	Concentration of antigen ($\mu g/10^7$ cells)	No. of experiments	Intensity of erythema	Diameter of erythema (mm)	Induration (mm increase in skin thickness)
Lymph node	–		0·37 (0–1)	4·07 (0–11)	3·68 (1·5–7)
	0·2	29	0·55 (0–2)	6·07 (0–12)	4·75 (1–12)
	10		1·09 (0·5–2)	11·00 (5–15)	8·24 (1·5–16)
Peritoneal lymphocytes	–		0·55 (0–2)	6·20 (0–14)	4·06 (1–85)
	0·2	29	0·85 (0·5–1)	9·05 (5–13)	7·32 (3·5–14)
	10		1·56 (0·5–3)	13·65 (6–17)	11·48 (5–23)
Peritoneal macrophages	0		1·12 (0–2)	6·25 (0–14)	5·25 (3·5–10)
	0·2	4	1·62 (0–3)	7·25 (0–17)	5·50 (1·5–10)
	10		2·25 (1–3)	12·00 (9–15)	7·75 (2–10·5)
Whole peritoneal exudate	0		1·41 (0–3)	9·91 (0–15)	5·95 (1·5–10·5)
	0·2	12	1·75 (0·5–3)	12·00 (8–14)	7·75 (2·5–18·5)
	10		2·16 (1–3)	14·08 (11–18)	8·41 (2·5–14)

In each experiment 0·1 ml of material from one culture consisting of pooled cells from ten donors, was tested in two to four guinea-pigs. Mean values and range (in parentheses) of inflammation parameters, as measured at 3 hours are shown for each type of supernatant.

testing, but the reaction is still evident at 24 hours. The presence of haemorrhage was a very rare event. No such activity was ever found in supernatants of normal lymphocytes (originating from animals injected with Freund's incomplete adjuvant) incubated with antigen or in cultures of lymphocytes from CFA immunized animals incubated with an unrelated antigen, namely ovalbumin. Active material could be obtained after 16 and 24 hours of culture but supernatants from 8-hour cultures were mostly inactive. Table 1 compares the mean numerical values for erythema and induration found by testing supernatants of lymph node cells, peritoneal lymphocytes, peritoneal macrophages and of the unfractionated peritoneal exudate population.

It is evident that the magnitude of the three chosen parameters of inflammation is dependent on the type of cells in culture and of the antigen cell number ratio.

Reactions are maximal with all cell types at a 10 μg PPD/10^7 cells ratio but peritoneal lymphocytes appear more active than LN cells, considering the fact that even though the latter are cultured at a five-fold cell concentration, LN supernatants were still the less active. No increase in skin activity was obtained by antigen supplementation of supernatants originating from cultures without antigen addition, a procedure which was nevertheless routinely performed.

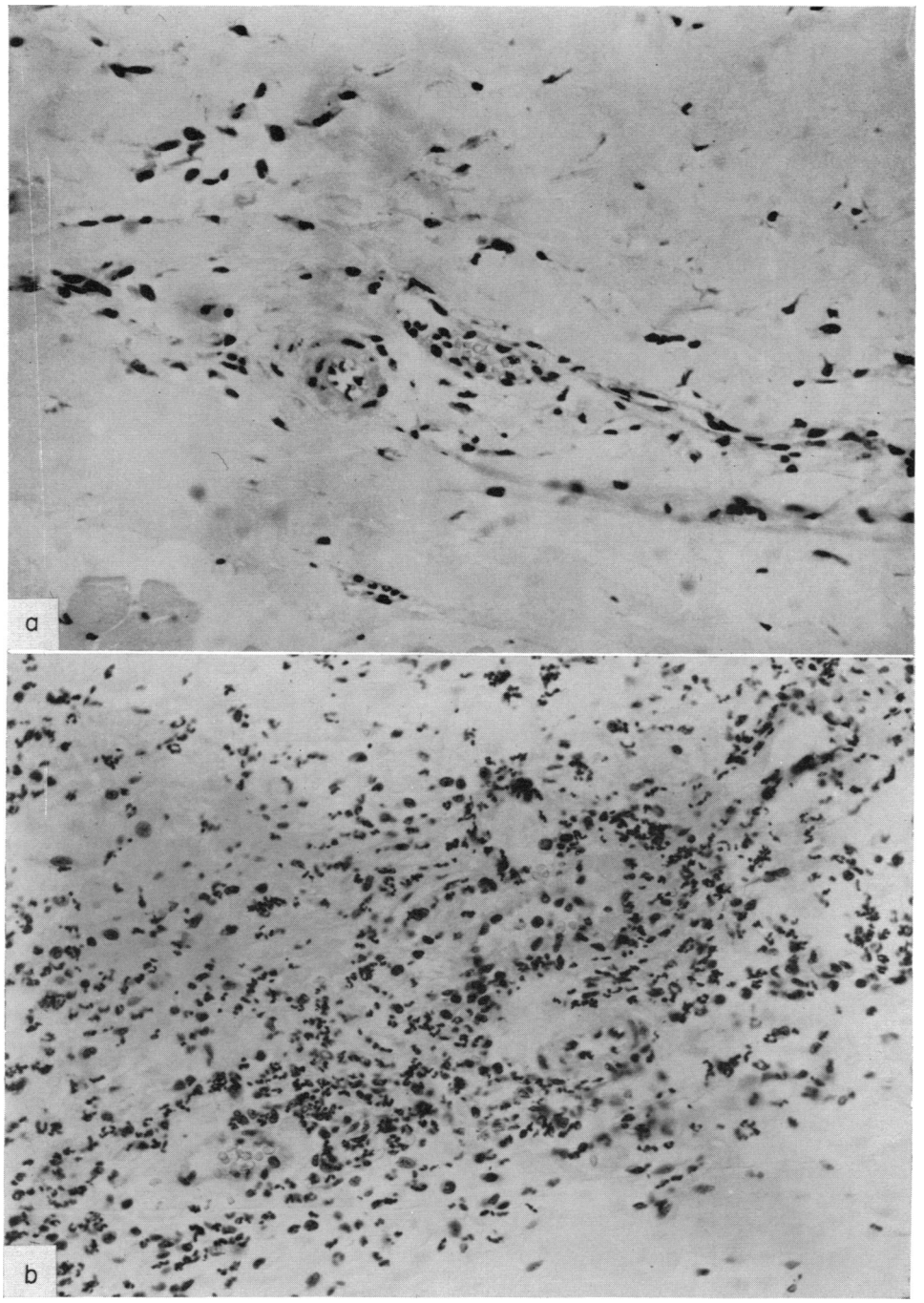

FIG. 1. Skin of a normal guinea-pig 6 hours after the intradermal injection of 0·1 ml of supernatant of sensitized PEL cultured: (a) without PPD; (b) in the presence of PPD, 10 $\mu g/10^7$ cells. The PEL are of the same batch as those used in the test illustrated in (a). Area between dermis and *panniculus carnosus*. H & E, ×200.

No potentiation of inflammatory activity could likewise be detected in supernatants of LN and PEL cultured with 0·2 μg PPD/10^7 cells when 50 μg PPD/ml (for LN) and 10 μg PPD/ml (for PEL) were added to the supernatant.

Unfractionated peritoneal exudates and even purified peritoneal macrophages were found to be able to give rise to an active supernatant in the presence of antigen but material derived from cultures without addition of antigen also possessed a rather strong inflammatory property which was mostly expressed by erythema but with little or no induration. Increasing the percentage of lymphocytes increased the antigen specificity, amplified the element of induration and reduced the intensity of erythema in the skin test. Compared to unfractionated peritoneal exudate cultures the erythematous element was also minimal in supernatants of LN cells, a population in which macrophages were practically absent.

Microscopically the 6-hour skin reaction induced by PPD incubated sensitized LN, PEL and peritoneal macrophages culture supernatants was characterized by a mixed polymorphonuclear–mononuclear infiltrate in the deeper dermis with a mainly perivascular localization (Fig. 1). The ratio of the two cell types was roughly 1 : 1 or showed a predominance of polymorphonuclears and in general resembled the local passive cellular transfer reactions as described by Blazkovec, Sorkin and Turk (1965). The 24-hour reaction had a similar aspect although the extent of cellular infiltration was somewhat reduced. No characteristic histology could be ascribed to supernatants of any particular cell type in culture.

SKIN ACTIVITY OF CELL EXTRACTS

Injection of 0·1 ml cell extract induced a mild inflammatory reaction with a maximum between 3 and 6 hours, with both erythema and induration being less than that following testing with supernatant (Table 2).

TABLE 2

SKIN INFLAMMATORY REACTION INDUCED BY EXTRACTS OF SENSITIZED LN CELLS AND PEL INCUBATED WITH SPECIFIC ANTIGEN

Origin of cell extract	Concentration of antigen in culture (μg/10^7 cells)	No. of experiments	Intensity of erythema	Diameter of erythema (mm)	Induration (mm increase in skin thickness)
Lymph node	–		0·42 (0–1)	3·85 (0–10)	2·35 (0–5·5)
	0·2	14	0·54 (0–1)	4·58 (0–10)	3·29 (1–7)
	10		0·60 (0·2)	5·00 (0–14)	2·85 (0·5–6·5)
Peritoneal lymphocytes	–		0·87 (0–2)	5·75 (0–14)	5·37 (0·5–10)
	0·2	4	0–37 (0–0·5)	2·00 (0–3)	1·25 (1–2)
	10		0·50 (0–1)	4·25 (0–8)	2·62 (1–5·5)

In each experiment 0·1 ml extract from one culture, consisting of cells from ten donors, was tested in two to four guinea-pigs. Numbers represent mean values and range.

Extracts of LN cells incubated with 0·2 μg PPD/10^7 cells had more inflammatory activity than control extracts but extracts obtained from cells incubated with 10 μg PPD showed little or no further increase in activity. Antigen exposure of peritoneal lymphocytes resulted in a decrease in inflammatory activity of extracts, representing a mirror image effect of the antigen dependent increase in activity seen in culture supernatants of the same cells.

The microscopical picture was identical to that seen with supernatants, with a clear predominance of polymorphonuclears. The intensity of the infiltrate induced by the injection of PEL extracts closely reflected the macroscopic measurements, antigen exposed cell extracts giving rise to a less extensive infiltrate than control extracts of cells cultured without antigen.

ANTIGEN-DEPENDENT MACROPHAGE CLUMPING

Skin activity similar to that described in the previous paragraphs could be obtained when sensitized cells were cultured in medium containing 20 per cent normal guinea-pig serum. When unfractionated peritoneal exudate cells were cultured in this medium, attachment of macrophages to the bottom of the incubation flasks was evident at the end of 24 hours. We found that the pattern of this macrophage sheet was strictly dependent on the presence and concentration of antigen in the medium. As it appears from Fig. 2 macrophages formed a uniform layer on the flask surface, when no antigen was present and showed an increasing degree of clumping with increasing antigen concentration. No

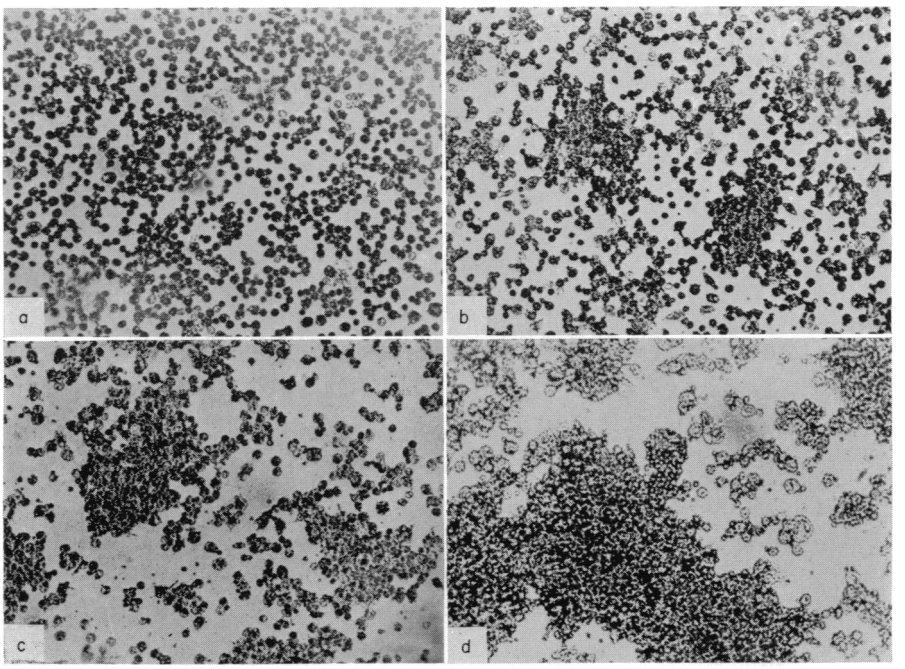

FIG. 2. Antigen induced clumping of macrophages from sensitized guinea-pigs. (a) No PPD present (b) 0·2 μg PPD/10^7 peritoneal exudate cells, (c) 10 μg PPD/10^7 cells, and (d) 25 μg PPD/10^7 cells. Pattern obtained after 24 hours in culture.

PPD induced clumping was observed with peritoneal exudates obtained from guinea-pigs injected with Freund's incomplete adjuvant or in cultures of immune peritoneal exudates in serum-free Carbowax containing medium. It is also significant that when purified macrophages obtained from immunized guinea-pigs were exposed to PPD, no clumping was seen. Although such cell populations are not entirely free of contaminating lymphocytes, it appears that the amount present is below that needed for the clumping effect to occur. A similar phenomenon has very recently been described in human peripheral blood cultures taken from tuberculin-sensitive donors (Hughes, 1968).

EFFECT OF INHIBITORS ON THE FORMATION AND ACTION OF SKIN REACTIVE SUPERNATANTS

The effect of Actinomycin-D, Puromycin and Chloramphenicol added to sensitized LN and PEL cultures on the appearance of skin activity in the supernatant, under the influence of antigen is shown in Table 3. At the concentration employed, puromycin was found to reduce the incorporation of [^{14}C]leucine into LN and PEL cell extract protein and the release of labelled protein into the supernatant to less than 5 per cent of the control value.

In order to examine the possibility that a proteolytic process is involved in the formation of a skin active component, two inhibitors of proteolysis were added to the culture: ε-aminocaproic acid and Soya bean trypsin inhibitor. ε-Aminocaproic acid at a concentration of 0·03 M or higher was reported to inhibit the primary binding and precipitation of antigen by antibody in addition to its ability to stop plasminogen activation by several enzymes (Wold, Reid and Farr, 1967).

TABLE 3

INFLUENCE OF INHIBITORS ON THE FORMATION OF SKIN ACTIVE CULTURE SUPERNATANTS

Type of cell in culture	Inhibitor	Concentration in medium (μg/ml)	Effect on skin activity	
			Erythema	Induration
Lymph node	Actinomycin-D	2	Reduced	Reduced
	Chloramphenicol	50	Reduced	No effect
	Puromycin dihydrochloride	50	Reduced	Inhibited
Peritoneal exudate lymphocytes	Actinomycin-D	2	Reduced	Reduced
	Chloramphenicol	50	No effect	No effect
	Puromycin dihydrochloride	50	Inhibited	Inhibited

Trypsin inhibitors were also found to inhibit the formation of pyrogenic material following the addition of specific antigen to sensitized cell extracts (Johanovsky and Skvaril, 1962). Addition of ε-aminocaproic acid (up to 0·075 M) and of Soya bean trypsin inhibitor (5 and 50 μg/ml) to LN and PEL cultures had no effect on the formation of the skin reactive factor. This lack of effect was evident in both serum-containing and serum-free Carbowax medium.

Injection of 1 ml anti-lymph node extract antiserum absorbed with guinea-pig red cells, intravenously, immediately preceding the intradermal injection of active LN or PEL supernatant resulted in a marked reduction of both the macroscopic and microscopic

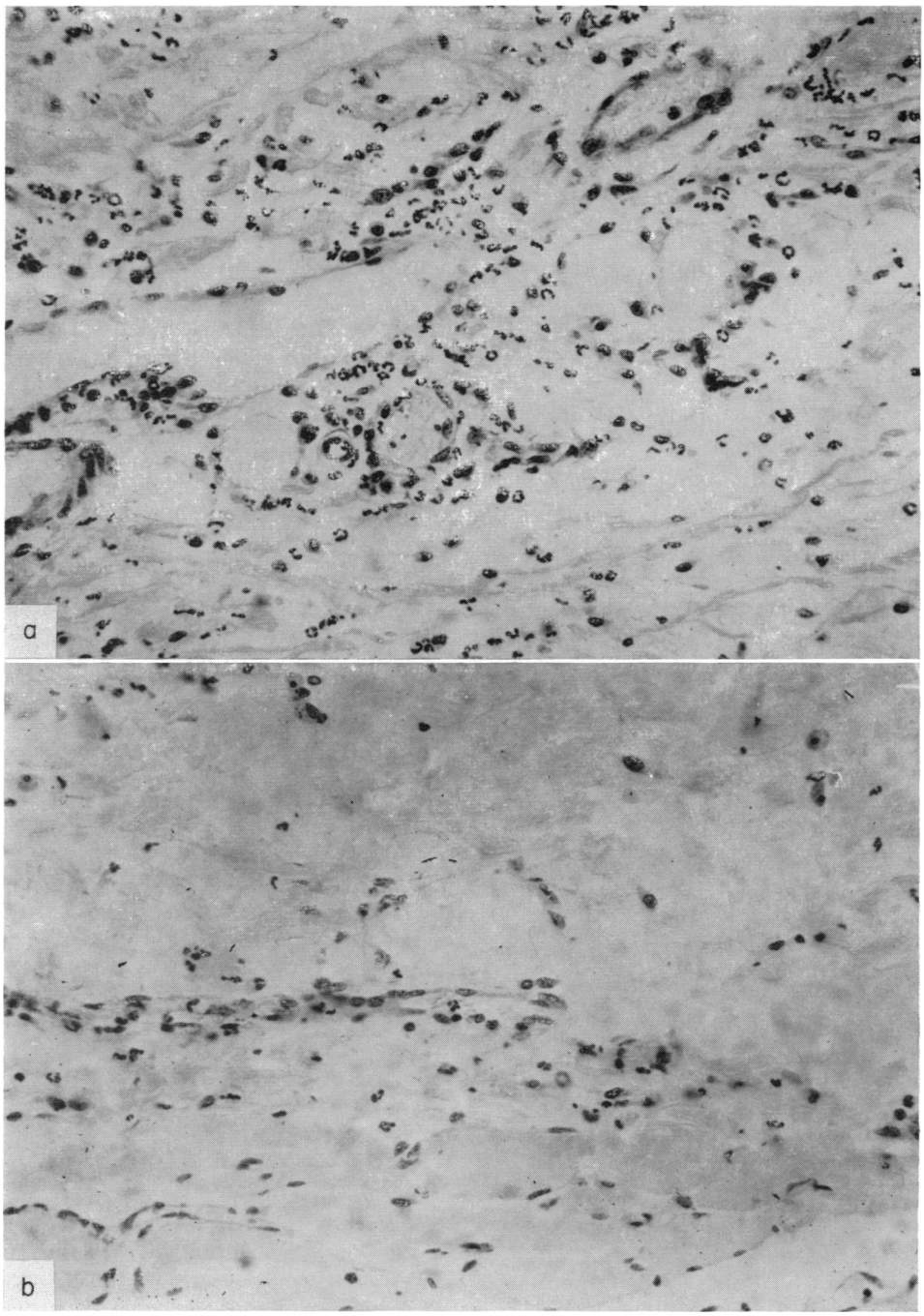

FIG. 3. Skin of: (a) a normal guinea-pig; and (b) a normal guinea-pig pretreated with ALNEx serum, 24 hours after the intradermal injection of 0·1 ml of supernatant of sensitized whole peritoneal exudate cultured in the presence of PPD, 10 $\mu g/10^7$ cells. Area between dermis and *panniculus carnosus*. H & E, ×200.

aspects of the inflammatory reaction. The comparative histology of the skin inflammation induced by PPD incubated sensitized peritoneal exudate culture supernatant in normal and ALNEx treated guinea-pigs is illustrated in Fig. 3. This effect was very similar to that described by Willoughby *et al.* (1965) on contact sensitivity in actively immunized animals and by Turk and Polák (1968) on the local passive transfer of the tuberculin reaction.

IMMUNOELECTROPHORETIC ANALYSIS OF CULTURE SUPERNATANTS

Serum-free supernatants from LN and PEL cultures with and without addition of PPD were analysed by immunoelectrophoresis using AGPS, ALNEx and ALS in the troughs. Two precipitin lines with a slow α and γG mobility were obtained by reacting a fifty-fold concentrated LN supernatant with all three antisera. An additional line with a fast α mobility was seen with ALNEx only. No lines were obtained with diluted material (Fig. 4). PEL supernatants without prior concentration constantly showed a single, intense precipitation line in the slow α region which could be developed with all three antisera but was most prominent with ALS and ALNEx (Fig. 4). This line stained positively for glycoprotein and for peroxidase by the *p*-phenylendiamine-α naphthol reaction but not by the benzidine test. No staining was obtained for lipid and nucleoprotein.

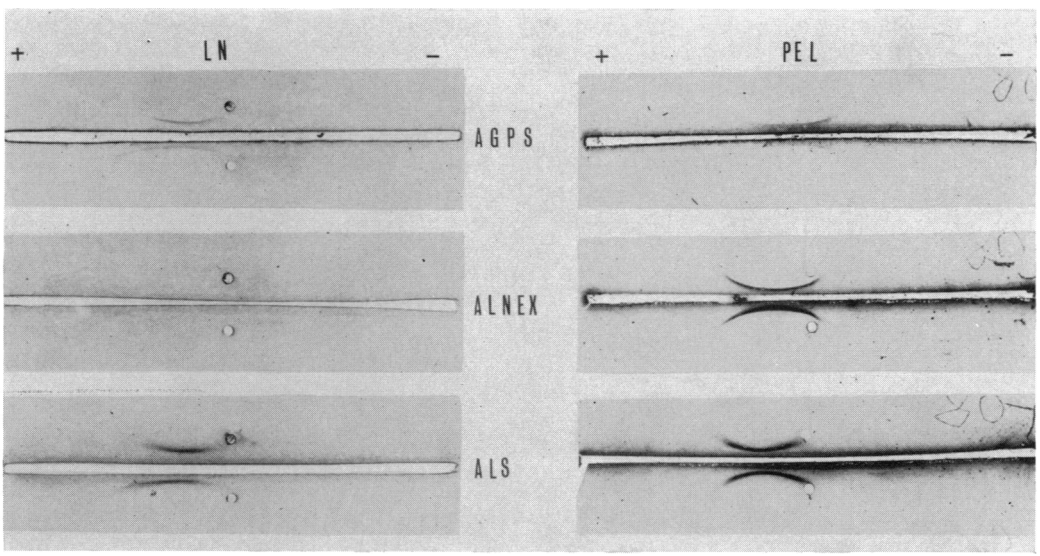

FIG. 4. Immunoelectrophoretic analysis of sensitized LN and PEL culture supernatants. Upper well contains concentrated material from cultures with addition of 0·2 μg PPD/10^7 cells, lower well material from cultures with addition of 10 μg PPD/10^7 cells. AGPS, Rabbit anti-whole guinea-pig serum; ALNEx, rabbit anti-guinea-pig lymph node extract; ALS, rabbit anti-guinea-pig lymphocyte serum.

Occasional PEL samples also possessed a γG line, the presence of which was dependent on the thoroughness of washing the cells before culturing them in serum-free medium.

No difference was found in the precipitation pattern and no additional line could be found when antigen incubated culture supernatants were compared to control material from culture without antigen. Absorption of ALS with freeze-dried guinea-pig serum

eliminated its capacity to precipitate the α_2 line in PEL supernatants. No precipitin lines were seen in immunoelectrophoresis of LN or PEL supernatants using anti-guinea-pig γM, α_2M and β_{1C} antisera.

FRACTIONATION OF ANTIGEN INCUBATED CULTURE SUPERNATANTS

Skin activity of supernatants was maintained after repeated freezing and thawing, after freeze-drying and redissolving and after prolonged dialysis against PBS at 4°. During dialysis a whitish precipitate was seen appearing, especially in LN samples but removal of the sedimented material by centrifugation did not diminish the activity of the supernatant. Dialysates of active supernatants did not possess any skin activity.

GEL FILTRATION ON SEPHADEX G-200

When concentrated LN supernatants obtained from cultures containing 10 μg PPD/10^7 cells were applied on Sephadex G-200, four peaks were obtained, when optical density was read at 215, 260 and 280 μ. The first three peaks corresponded to that of macroglobulin, 7S globulin and albumin, fractionated on the same column, the last one was of small molecular weight (Fig. 5). When peaks were freeze-dried, redissolved to 20 per cent of the original volume of the sample applied on the column, dialysed against PBS and tested in the skin, activity was found only in Peaks III and IV and was maximal in Peak III. Immunoelectrophoretic analysis of highly concentrated peaks using AGPS and ALS

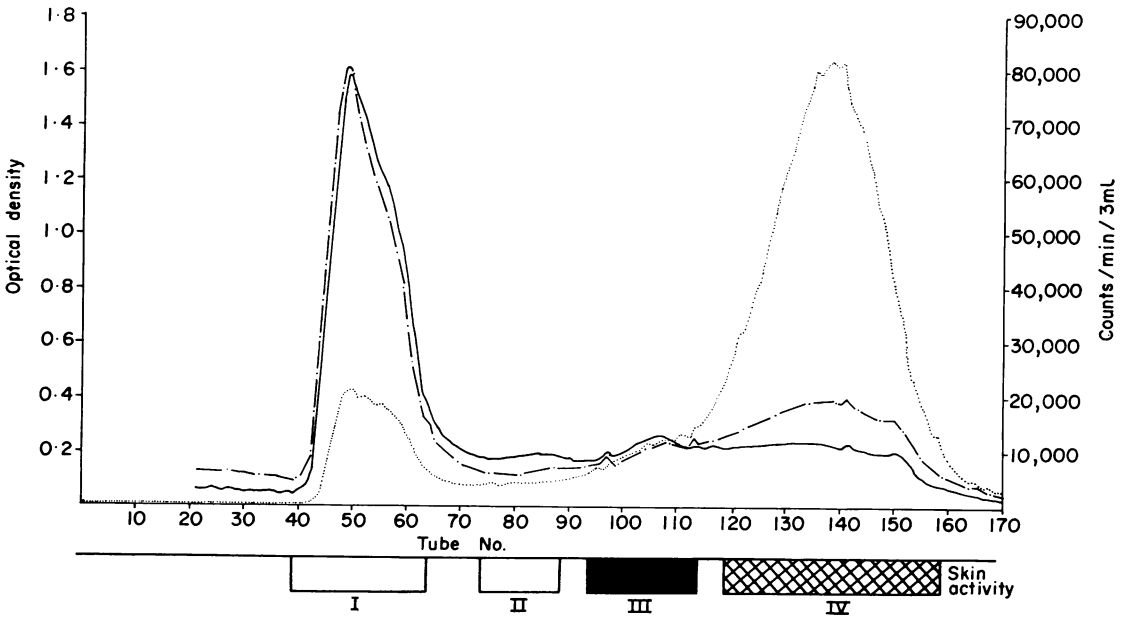

FIG. 5. Sephadex G-200 fractionation of supernatant from a culture of sensitized LN cells incubated with [^{125}I]PPD, 10 μg/10^7 cells, concentrated from 225 ml. Column, 2·5 × 100 cm and elution with PBS at 9 ml/hr. Volume of each fraction was 3 ml. Elution patterns show OD at 280 mμ (——), OD at 260 mμ (—·—·) and amount of [^{125}I]PPD as counts/min 3 ml sample (.....). Fractions in each of the four peaks were pooled and skin activity is indicated schematically by the degree of shading of rectangles representing these peaks. Peak III contained maximum skin activity, Peak IV, moderate skin activity, Peaks I and II were inactive.

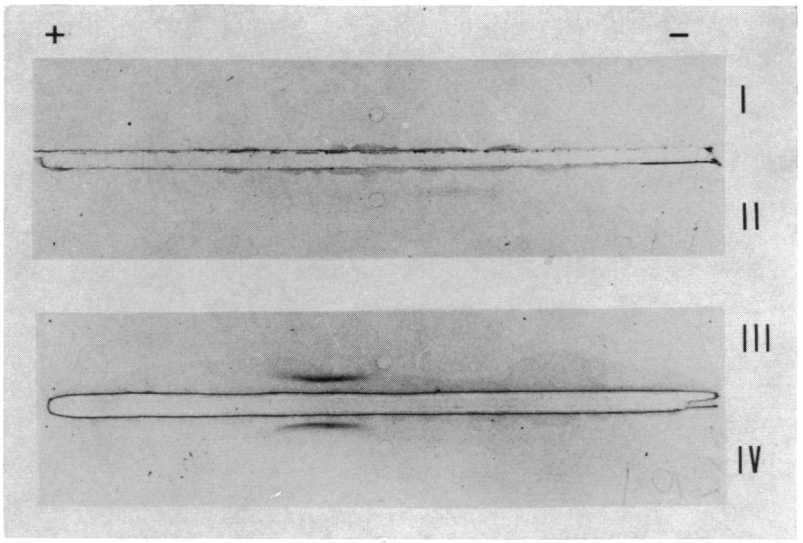

FIG. 6. Immunoelectrophoretic pattern of LN culture supernatant fractionated on Sephadex G-200. Wells contain Peaks I, II, III, and IV and ALS was used in the troughs. Only Peaks III and IV possessed skin activity. Note presence of slow α line in these two peaks.

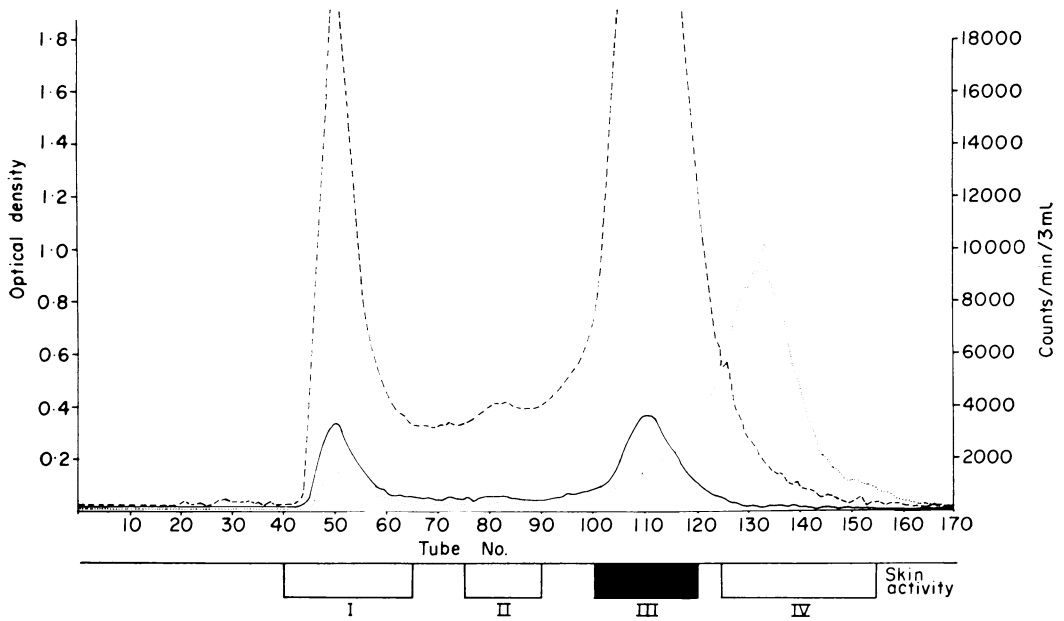

FIG. 7. Sephadex G-200 fractionation of supernatant from a culture of PEL incubated with [^{125}I]PPD, 10 μg/10^7 cells, concentrated from 50 ml. Column, 2·5 × 100 cm and elution with PBS at 9 ml/hr. Volume of each fraction was 3 ml. Elution patterns show OD at 215 mμ (- - -) OD at 280 mμ (——) and amount of [^{125}I]PPD as counts/min/3 ml sample (.....). Fractions in each of the four peaks were pooled and skin activity is indicated schematically by the degree of shading of rectangles representing these peaks. Skin activity was limited to the material in Peak III.

showed the presence of a γG line in Peak II, of a slow α line in Peaks III and IV and no precipitin line in Peak I (Fig. 6). When ALNEx was used in the trough a γG precipitin line was obtained with Peak II, two faint α lines of slow and fast mobility with Peak III and no precipitin line with Peak IV. Fractionation of PEL supernatants from cultures incubated with 10 μg PPD/10^7 cells on Sephadex G-200 resulted in three peaks, also corresponding to macroglobulin, 7S γ-globulin and albumin (Fig. 7). Skin activity was limited to Peak III. Immunoelectrophoretic analysis showed the α_2 line, seen in whole PEL supernatants and precipitated by AGPS, ALS and ALNEx, to be present in Peak III only (Fig. 8a). No precipitin lines were observed with Peaks I and II.

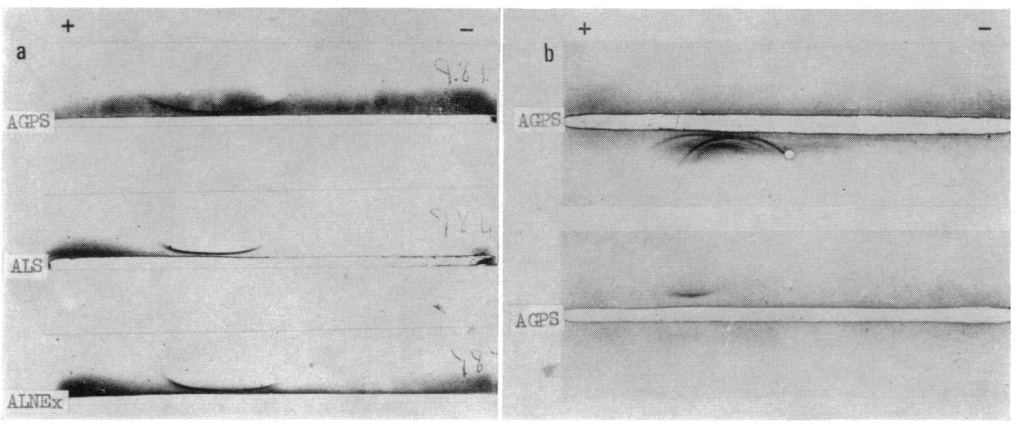

FIG. 8. Immunoelectrophoretic patterns of column fractionated PEL supernatant. (a) All the wells contain the skin active Sephadex G-200 Peak III. (b) Sephadex G-200 Peak III was refractionated on DEAE-cellulose. Wells contain, from top to bottom, Peaks I–IV obtained by gradient elution. Peak II was most active in the skin. AGPS, Rabbit anti-whole guinea-pig serum; ALS rabbit anti-guinea-pig lymphocyte serum; ALNEx, rabbit anti-guinea-pig lymph node extract. The presence of multiple α lines and of albumin in the DEAE-cellulose peaks, while being absent in the original Sephadex G-200 Peak III, is explained by the higher degree of concentration to which DEAE-cellulose peaks were submitted, preceding immuno-electrophoresis.

LOCALIZATION OF PPD IN SEPHADEX G-200 FRACTIONS OF SUPERNATANTS

LN cells and PEL were incubated as described under 'Materials and methods' but using [^{125}I]PPD (specific activity 0·018 μc/μg) as antigen. Supernatants obtained from radioactive antigen incubated cultures possessed the usual antigen specific skin activity.

Supernatants from cultures containing 10 μg [^{125}I]PPD/10^7 cells were concentrated by freeze-drying, dialysed for 72 hours against PBS and fractionated on columns of Sephadex G-200. The effluent fractions were read for optical density and counted for radioactivity in a γ counter. As it can be seen from Figs. 5 and 7, PPD appears in two major peaks, in both LN and PEL cultures, the first overlapping the macroglobulin peak, the second corresponding to Peak IV in LN supernatants and following Peak III in PEL supernatants. Peak III of both LN and PEL material also contains a certain amount of radioactivity. We can, therefore, conclude that there is no direct relationships between skin activity and antigen content of Sephadex peaks but at the same time all active fractions contain a certain amount of PPD.

RADIOIMMUNOELECTROPHORETIC ANALYSIS OF [^{125}I]PPD INCUBATED CULTURES AND THEIR SEPHADEX FRACTIONS

Whole supernatants and Sephadex peaks were concentrated by vacuum dialysis to volumes of 0·1–0·2 ml and analysed by immunoelectrophoresis followed by exposure on X-ray film. Four different antisera were added to the troughs: AGPS, ALS, ALNEx and anti-guinea-pig γG.

A radioactive line was detected only on the slide containing LN supernatant from cultures with 10 μg [^{125}I]PPD/10^7 cells in the well and anti-γG in the trough (Fig. 9a). The very same line was detected in Peak III of the Sephadex G-200 fractionated [^{125}I] PPD incubated LN culture supernatant, using anti-guinea-pig γG serum (Fig. 9b). No radioactive precipitin line was observed when AGPS, ALS or ALNEx were used as antisera.

No line could be detected by staining the same slides with Amido Black and the identity of the [^{125}I]PPD binding material, precipitated by anti-γG remains as yet unknown.

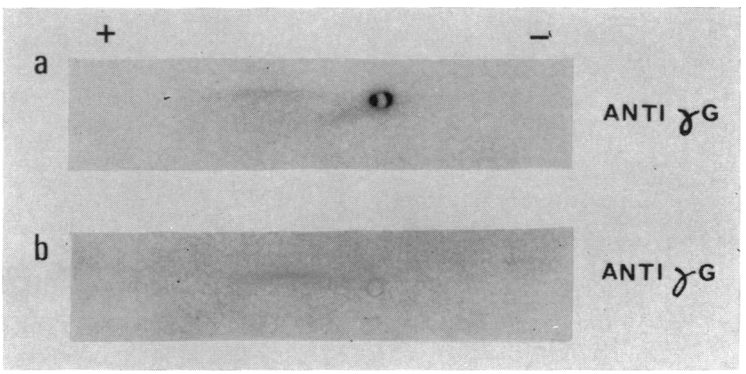

FIG. 9. Radioimmunoelectrophoresis of the supernatant of a sensitized LN culture containing [^{125}I] PPD (10 μg and 0·19 μc/10^7 cells). (a) Whole supernatant in the well, anti-γG serum in the trough. (b) Peak III of Sephadex G-200 fractionated supernatant in the well. Anti-γG serum in the trough. This peak contains the skin reactive material.

FRACTIONATION OF SKIN ACTIVE SEPHADEX G-200 PEAKS ON DEAE-CELLULOSE

Peak III from skin active LN or PEL supernatants was applied to columns of DEAE-cellulose and eluted with succession of 0·01 M potassium phosphate buffer, pH 8·0, followed by a 0·01–0·3 M linear gradient of the same buffer. LN supernatant Sephadex G-200 Peak III resulted in four peaks on DEAE-cellulose, the first one being eluted with the starting buffer, the other three with the gradient (Fig. 10). Skin activity was found in Peak II (the first gradient peak). No precipitation line was detected in any of the LN, DEAE-cellulose peaks by IEP.

PEL supernatant Sephadex G-200 Peak III also resulted in four peaks on DEAE-cellulose; the first one being eluted at 0·01 M, the other three during the gradient (Fig. 11).

Skin activity was maximal in Peak II, although some inflammation was caused by Peaks III and IV too. Several precipitin lines with a slow α mobility were detected by IEP in Peak II but only when AGPS was used as antiserum and small amounts of serum albumin were found in Peaks III and IV (Fig. 8b).

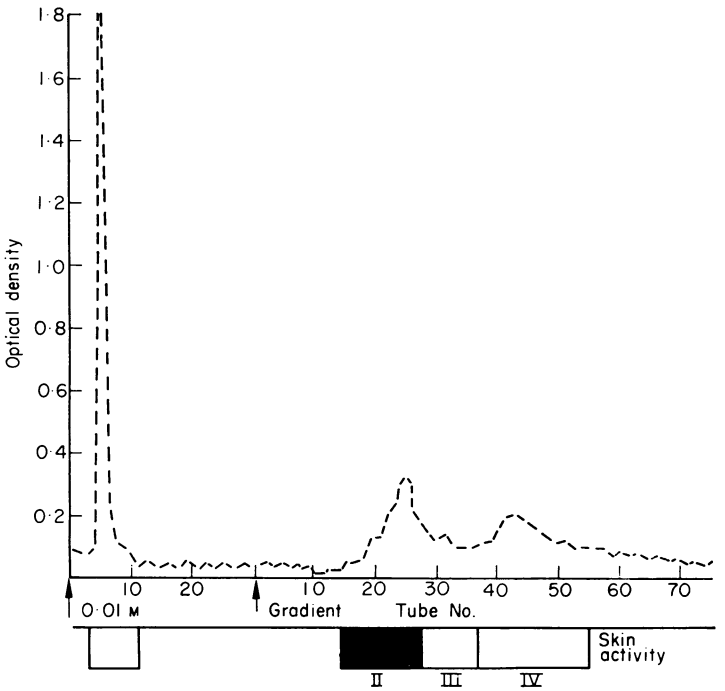

FIG. 10. DEAE-cellulose chromatography of Peak III obtained by gel filtration on Sephadex G-200 of PPD incubated LN culture supernatant (10 μg PPD/10^7 cells). Columns were 1·5 × 45 cm and elution was performed at 24 ml/hr. Eluants were 0·01 M potassium phosphate buffer, pH 8·0, followed by a linear gradient (0·01–0·3 M) of the same buffer. The starting points of the eluants are indicated by arrows. The volume of each fraction was 5 ml. Chromatographic pattern shows OD at 215 mμ (- - - -). Fractions in each of the four peaks were pooled and skin activity expressed by the shading of rectangles representing these peaks. Only Peak II contained skin reactive material. Peaks I, III and IV were inactive.

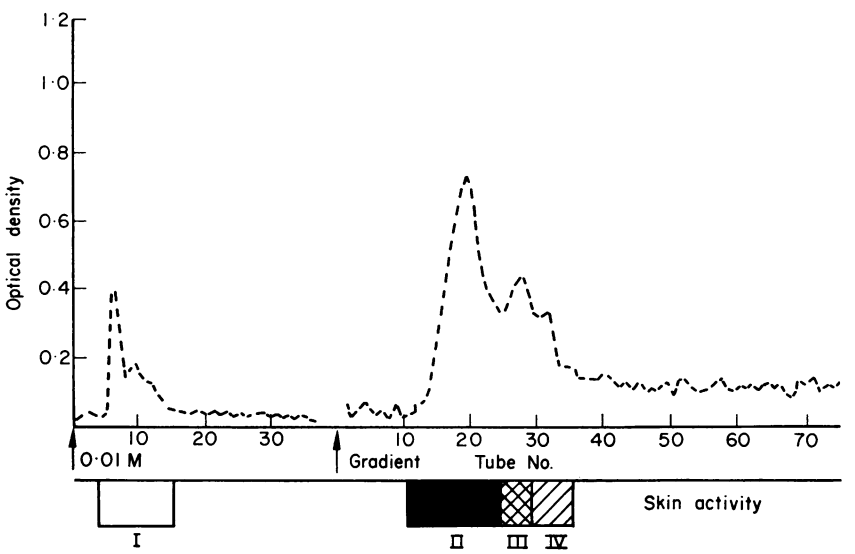

FIG. 11. DEAE-cellulose chromatography of Peak III obtained by gel filtration on Sephadex G-200 of PPD incubated PEL culture supernatant (10 μg PPD/10^7 cells). Conditions as Fig. 10. Peak II had maximal activity, Peak III, moderate and Peak IV a trace of skin activity. Peak I was inactive.

Chromatography on DEAE-cellulose therefore allows subfractionation of the non-homogenous Peak III from Sephadex G-200, into four peaks only one of which contains most or all of the skin active material. It is also clear that on gradient fractionation the active material is eluted after γ_2G but before serum albumin. Its association or identity with one of the α-globulins detected in Peak II remains an open question.

ANTIGEN BINDING BY CULTURE SUPERNATANTS

Binding of ^{125}I-labelled PPD by supernatants and cell extracts of sensitized LN and PEL cultured without antigen or in the presence of 0·2 μg and 10 μg unlabelled PPD/10^7 cells, was examined by radioimmunoelectrophoresis. No radioactive precipitin lines could be detected by this method, in which the labelled antigen was added to the trough, following immunoelectrophoresis. This result is in contrast with the positive binding seen when labelled PPD is present in the culture medium during incubation, as described earlier.

TREATMENT OF CULTURE SUPERNATANTS WITH ENZYMES

As it appears from Table 4 incubation of both LN and PEL supernatants with pepsin completely abolished their inflammatory activity. Some reduction was affected by mere incubation for 16 hours at 37° and at a pH of 4·1, without the addition of pepsin. Trypsin digestion was much less effective than pepsin although clear depression especially of the erythema inducing activity, was evident. Control incubation for 6 hours at 37° and pH 7·6 had no effect. Papain was, if anything, less effective than trypsin and clearly less effective than pepsin in decreasing the inflammatory activity while control incubation for 16 hours at pH 7·0 slightly impaired the inflammatory potency of PEL supernatants only.

TABLE 4

INFLUENCE OF PROTEOLYTIC ENZYMES ON SKIN ACTIVITY OF CULTURE SUPERNATANTS

	LN-supernatant			PEL-supernatant		
	Intensity of erythema	Diameter of erythema (mm)	Induration (mm increase in skin thickness)	Intensity of erythema	Diameter of erythema (mm)	Induration (mm increase in skin thickness)
Pepsin (250 μg/ml, 16 hours)						
Control, 4°	0·75	10·00	9·50	1·37	13·00	14·25
Control, 37°	0·50	6·00	5·50	0·50	11·00	8·50
Pepsin	0	0	3·25	0	0	1·75
Trypsin (insoluble enzyme, 10 mg/ml, 6 hours)						
Control, 4°	0·50	9·50	4·75	0·75	12·00	9·25
Control, 37°	0·50	9·00	5·50	0·75	13·50	9·50
Trypsin	0	0	4·75	0·25	5·50	4·50
Papain (insoluble enzyme, 10 mg/ml, 16 hours)						
Control, 4°	0·50	10·00	4·75	1·00	14·00	10·00
Control, 37°	0·50	8·00	6·00	0·50	10·50	8·50
Papain	0·25	4·00	7·00	0·75	8·00	7·25

Figures represent mean values obtained in four test animals. Control samples were dialysed, incubated and centrifuged as experimental samples but with omission of the enzyme.

Immunoelectrophoretic analysis of enzyme digested PEL supernatants revealed the total disappearance of the slow α line in the pepsin digested samples, its partial destruction by papain and its persistence following trypsin treatment. Treatment of culture material with ribonuclease and deoxyribonuclease had no effect on its skin activity.

AMMONIUM SULPHATE PRECIPITATION OF LN AND PEL CULTURE SUPERNATANTS

Supernatants of sensitized LN and PEL cultures incubated with 10 μg PPD/10^7 cells were precipitated with ammonium sulphate at a final concentration of 40, 46, 53, 60 and 66 per cent saturation. Supernatants and redissolved precipitates dialysed free of ammonium sulphate were tested in normal skin. At 40 per cent saturation all skin activity was found in the supernatant of both LN and PEL material. At 66 per cent saturation inflammatory activity was found exclusively in the precipitate of PEL material, while LN culture precipitates contained most but not all the skin activity, some inflammation also being caused by the supernatant fraction. Saturations of 46, 53 and 60 per cent did not give clear cut results since both the precipitate and the supernatant possessed skin activity but a tendency towards more activity in the precipitate with increasing degrees of saturation was apparent.

The fact that not all the inflammatory activity was recovered in the precipitate of LN supernatants, even at 66 per cent saturation was probably due to the lower total protein content of these supernatants as compared with PEL material.

ANTIGEN INDUCED SPECIFIC PROTEIN SYNTHESIS IN LN AND PEL CULTURES

Immune LN cells and PEL were incubated for 24 hours without antigen and in the presence of 0·2 and 10 μg PPD/10^7 cells, in conditions strictly similar to those which were used in order to obtain skin active supernatants but in leucine-free Eagle's medium, to which [^{14}C]leucine was added at a concentration of 1 μc/ml. At the end of the culture,

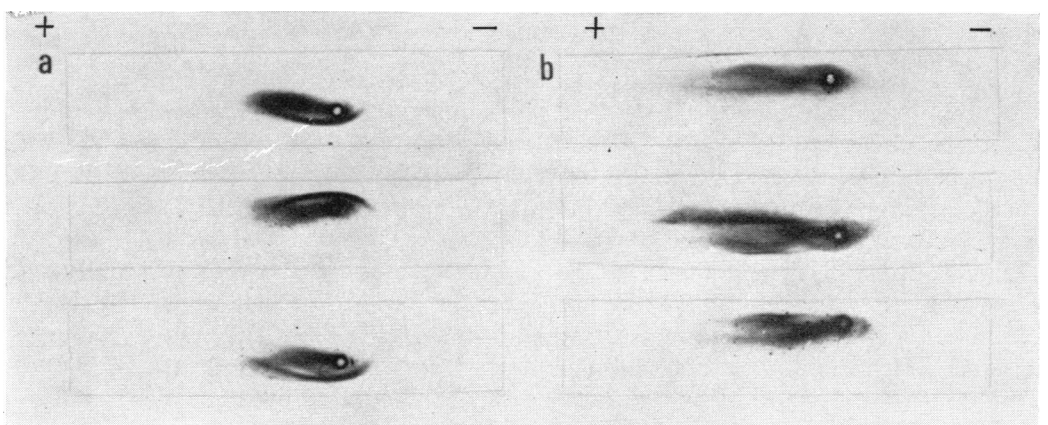

FIG. 12. Radioimmunoelectrophoretic patterns of sensitized whole peritoneal exudate culture supernatant (a) and cell extract (b) following incorporation of [^{14}C]leucine *in vitro*. Upper wells, No PPD added; centre wells 0·2 μg PPD/10^7 cells; lower wells 10 μg PPD/10^7 cells. The antiserum in the troughs was ALNEx. PPD induces the synthesis and release into the supernatant of an α_2 and of an α_1 protein (a, centre and lower wells). An intense β line is seen both with and without antigen exposure. A slow α protein line can likewise be detected in the soluble cell extract, when PPD was present in the culture (b, centre and lower wells).

supernatants and cell extracts were concentrated by vacuum dialysis and dialysed free of non-protein [^{14}C]leucine. Care was taken during this procedure to bring the various samples to the same degree of concentration, so as to make a comparison possible. The highly concentrated extracts and supernatants were examined by radio-immunoelectrophoresis. LN cells and PEL from guinea-pigs injected with Freund's incomplete adjuvant were cultured in identical conditions and extracts and supernatants tested the same way.

Exposure of immune LN cells to 0·2 and 10 μg PPD/10^7 cells induced the synthesis and release into the supernatant of a fast α protein, which was precipitated by ALNEx serum. A similar line was also evident in antigen incubated PEL supernatants. It was found that extracts of PEL cultured without antigen contain the very same neosynthesized material precipitating as an α_1 protein and that exposure to PPD appears to progressively deplete the intracellular compartment of it and induce its release into the supernatant. When whole peritoneal exudates are incubated in the conditions described above, neosynthesis of an α_2 and of an α_1 protein and their release into the supernatant was evident (Fig. 12a). A strong β line which does not appear in PEL material is present in these supernatants independently of antigen exposure and possibly represents a macrophage product. The whole peritoneal exudate extracts are richer in lines than the purified lymphocyte extracts and antigen dependent synthesis of an α_2 protein is also evident (Fig. 12b). Since this protein is absent in extracts of exudate cells without antigen exposure it is probable that antigen induces its synthesis in the cultured cells, possibly macrophages, this being followed by its release into the supernatant. These results seem to indicate that at least two α proteins are synthesized and released into the skin active culture supernatant under the influence of antigen with a possible indication that one is a lymphocyte product (α_1, released by LN cells and PEL) the other belonging to the macrophages (α_2). The presence of Puromycin (50 μg/ml) completely inhibited the synthesis of both proteins.

Synthesis of γG could be detected in LN but not in PEL cultures, whether originating from CFA immunized or normal animals, and was not related to exposure to antigen.

RELEASE OF A SKIN REACTIVE FACTOR UNDER THE INFLUENCE OF PHYTOHAEMAGGLUTININ

Phytohaemagglutinin (PHA-P) was added to cultures of LN cells (5×10^7 cells/ml) and PEL (1×10^7 cells/ml), from CFA immunized guinea-pigs. The mitogen was added in concentrations of 0·1, 0·2 and 0·3 ml of a 1 : 30 dilution of the reconstituted PHA-P, to flasks containing 5 ml of culture. Controls containing cells without PHA and PHA alone as well as the experimental cell–PHA cultures were incubated for 24 hours after which supernatants were separated and tested in the skin.

Material from PHA incubated LN and PEL cultures induced a typical skin inflammation similar to that seen with specific antigen incubated cell supernatants. The degree of inflammation was nearly maximal with 0·1 ml PHA-P per flask, and was not augmented by increasing the amount of PHA in the culture. Injection of PHA-P dissolved in medium to the same dilution, and incubated for 24 hours at 37° caused little or no skin inflammation.

DISCUSSION

It can, therefore, be concluded that incubation of LN cells and PE lymphocytes from CFA immunized guinea-pigs, with tuberculin PPD, results in the release into the culture

supernatant of one or several factors able to induce inflammation upon intradermal injection in normal animals. Minimal amounts of such a factor are also detectable in antigen incubated immune LN cell sonicates. The nature of the skin inflammation only partially resembles the classical delayed reaction as seen in actively immunized animals but has many characteristics in common with the local passive transfer reaction.

It is our assumption that the *in vitro* phenomenon, we described, closely mirrors the *in vivo* events as they happen at the site of a delayed skin test but there is no proof at present that it is exclusively related to cellular hypersensitivity.

The formation of the skin reactive factor is strictly dependent on the state of immunity of the cell donors, on the ratio of antigen to cell number in the culture and on the duration of the culture. It is also important that PE lymphocytes were found to be much more potent in releasing the inflammatory material than LN cells from the same animals. This striking difference was also described in the local passive transfer of tuberculin hypersensitivity (Blazkovec *et al.*, 1965) and in the inhibition of macrophage migration (Dumonde *et al.*, 1968), two phenomena closely related to the state of delayed hypersensitivity. The formation of the skin active material was found to require an active synthetic process which is depressed or totally inhibited by Actinomycin-D and Puromycin, respectively. Puromycin inhibits protein synthesis in mammalian cells by impairing the binding of amino acids to specific transfer RNA and, therefore, causing a premature release of the peptide chain from ribosomes (Nathans, 1964). Actinomycin-D combines with guanine containing sites of DNA and, therefore, inhibits DNA dependent RNA synthesis (Goldberg, Rabinowitz and Reich, 1962). Chloramphenicol influences protein synthesis by acting as an inhibitor of ribosome synthesis and was found to suppress the primary immune response in mammals (Weissberger, Daniel and Hoffman, 1964).

Our results with the inhibitory drugs are well in accordance with the reported suppressive effects of Mitomycin-C and Actinomycin-D on the systemic transfer of contact hypersensitivity in the guinea-pig (Bloom, Hamilton and Chase, 1964). The negative results with chloramphenicol are unexplained and might be due to special dosage requirements for the guinea-pig. A lack of effect of chloramphenicol, administered *in vivo*, on delayed skin reactions to diphtheria toxoid was recently reported (Graham and Hancock, 1968).

The requirement for an intact synthetic mechanism is also in good agreement with the fact that short term incubation (8 hours) is insufficient for the formation of detectable amounts of skin reactive factor.

The fact that serum-free medium was found to be equally well suited for allowing the appearance of activity as serum-containing medium, clearly demonstrates that the reactive material originates in the lymphocytes and is not derived from serum.

Of special interest is the role of different cell types in the formation of the factor, as illustrated by the different types of skin reactions obtained when supernatants of peritoneal lymphocytes, macrophages or whole exudates are tested. Although obviously an over simplification, it seems as if macrophages respond to antigen by releasing merely an erythema inducing substance and contribute little to the induration while the peritoneal lymphocytes react to antigen by releasing both erythema and induration inducing factors. The antigen reactive components of macrophages and lymphocytes were not investigated in this part of our work but the role of a cytophilic antibody in the macrophage response cannot be excluded. Preliminary work indicates that normal macrophages incubated with immune lymphocyte culture supernatants and subsequently exposed to antigen, respond by the release of an erythema inducing substance similar to that just described.

The formation of an active supernatant is strictly antigen specific and dose dependent and therefore strongly suggests a reaction between antigen and antibody or an antibody-like component on the lymphocyte. The fact that no activity was present after addition of PPD to supernatants of immune lymphocytes incubated without antigen demonstrates that a reaction between antigen and antibody normally released into the culture does not explain the phenomenon. The radio-immunoelectrophoretic evidence for the presence of antigen binding by LN supernatants makes the presence of an antigen–antibody complex probable, but its role in the release of skin reactive materials remains to be explained.

The inhibitory effect of ALNEx serum on the skin inflammation, induced by antigen incubated LN and PEL culture supernatants must be analysed in the light of the ability of this antiserum to suppress contact hypersensitivity (Willoughby *et al.*, 1965) local passive transfer of the tuberculin reaction (Turk and Polák, 1968) and the Arthus reaction (Turk and Polák, 1969). The almost total suppression of the supernatant induced inflammation seems to indicate that common mediators are involved in this and the other phenomena, mentioned above. Since ALNEx serum is produced by immunization with intracellular lymphocytic material, its inhibitory action would indicate that antigen exposure results in the release of one or several intracellular substances into the culture supernatant, which substance(s) act as effector(s) of the skin inflammation. That antigen is not solely causing a transfer of intracellular inflammatory material into the culture medium but also involves some other activating and probably synthetic mechanism is indicated by the slight but definite increase in activity which occurs with antigen incubated LN cell extracts.

Immunoelectrophoresis analysis of LN and PEL culture supernatants proved to be of little help since no qualitative or quantitative difference was found between antigen incubated and control culture supernatants. The positive peroxidase reaction and electrophoretic mobility of the α_2 line found in PEL supernatants suggest its identity with haemaglobin and its origin in the red cells contaminating the culture.

Fractionation of both LN and PEL supernatants on Sephadex G-200 resulted in concentration of skin activity in a peak with the molecular weight of serum albumin. A fourth peak of small molecular weight possessed skin activity in LN supernatants only. Refractionation of the active Peak III on DEAE-cellulose localized the skin activity in fractions eluted at the beginning of the 0·01–0·3 M gradient. At present it is impossible to decide whether the material precipitated by AGPS and ALS as an α_2 protein in Peaks III and IV of Sephadex fractionated LN supernatants or the two α proteins precipitated by ALNEx in Peak III only, have any relationships to the skin active factor.

As far as PEL supernatants are concerned, the slow α protein precipitated by all antisera and constantly present in the skin active Sephadex Peak III was identified as haemoglobin. Dissociation of activity from this contaminant was effected by chromatography on DEAE-cellulose and the active peak was found to contain several slow α lines distinct from haemoglobin and precipitated by AGPS only. The localization of the active material in our culture supernatant by Sephadex filtration confirms the findings of Bennett and Bloom (1968) and Dumonde *et al.* (1968) as obtained under different experimental conditions. The skin active substances appeared to be completely destroyed by pepsin digestion and partially destroyed by trypsin and papain while being totally insensitive to DNase and RNase. Incubation for 16 hours at 37° at pH 7 had little influence on the activity of the material but incubation for the same length of time pH 4·1 markedly reduced its potency. This might be explained by autodigestion by lymphocytic proteolytic enzymes

(cathepsins) present in the supernatant and which became activated at an acid pH. The lack of effect of proteolytic inhibitors on the formation of the skin reactive material fits well into this picture since it can be expected that their influence would rather increase the amount of active substance by preventing its digestion during the culture interval.

The inflammatory material is not precipitated by ammonium sulphate at 40 per cent saturation and can be detected in the supernatant following elimination of the salt by dialysis. At saturations higher than 50 per cent the activity accumulated progressively in the precipitate but total precipitation is attained only at 66 per cent saturation. These data would exclude antibody or antigen–antibody complexes of the classical type from being the actual phlogistic material. The rather high concentration required for precipitation seems to point towards a substance similar to α_1 glycoprotein. It can therefore be concluded that the factor present in antigen incubated immune LN and PEL supernatants is a non-dialysable substance of a molecular weight close to 70,000, most probably a protein or closely associated with a protein, sensitive to pepsin and precipitable with ammonium sulphate at 66 per cent saturation. It is possibly identical with or associated with an α_2-globulin which on chromatography on DEAE-cellulose is eluted after γ_2G-globulin but before serum albumin.

Experiments employing ^{125}I-labelled PPD demonstrate that the third Sephadex peak, which has maximal skin activity is poor in antigen, the bulk of PPD being eluted in the small molecular weight peak. Of special importance is the finding in the third Sephadex peak of PPD bound to a protein of unknown nature which is precipitated by anti-γG antiserum.

Considering the molecular weight of proteins eluted in the 'albumin' peak, the antigen binding properties and the capacity to be precipitated by anti-γG, the obvious candidate would be the H chains of γG, possibly secreted as such into the culture supernatant.

Direct evidence for an antigen directed synthetic process is brought by radioimmunoelectrophoresis with [^{14}C]leucine labelled culture supernatant. This indicates that antigen exposure of immune but not of normal lymphocytes, results in the specific synthesis of an α_1-globulin. It is possible that this material is normally synthesized by circulating lymphocytes but secreted into the culture medium under the influence of antigen. Addition of macrophages to peritoneal lymphocytes results in the synthesis of an α_2-globulin, when antigen is added. Whether this is the result of a direct effect of antigen on immune macrophages or is secondary to the PPD–lymphocyte interaction is unknown.

Of special interest is the finding that exposure of LN cells and PEL to PHA results in the release of a skin reactive material, with an effect indistinguishable from that produced by the supernatant of PPD incubated specifically immune lymphocytes. This means that PHA shares with specific antigen not only its mitotic, RNA and DNA synthetic properties, but also the ability to release a skin reactive factor from LN cells and PE lymphocytes. A recent report also demonstrates the PHA induced synthesis of a number of proteins in human peripheral lymphocytes, two of them being located in the α_1 and α_2 regions on radio-immunoelectrophoresis (Scheurlen, 1968). It remains to be determined whether the physico-chemical characteristics of the PHA released skin active material are identical with those of the PPD induced factor. We can, therefore, conclude that *in vitro* exposure to antigen of lymphocytes from guinea-pigs in a state of delayed hypersensitivity to PPD results in the release into the culture medium of a substance able to induce a pronounced skin inflammation when tested in normal animals. This reaction has several characteristics in common with the classical delayed skin test but it develops sooner and its

polymorphonuclear element is more extensive. This fact is hardly surprising considering the basic difference between a preformed substance which has been synthesized during the 24 hours of *in vitro* incubation and the progressive accumulation of a similar factor at the site of an *in vivo* skin test.

The condition of its formation, its enzyme sensitivity, its independence from serum proteins and its molecular weight correspond to that of the material inhibiting normal macrophage migration but the identity of the two factors remains to be proven. The supernatant fraction inducing skin inflammation does not contain antigen in significant amounts and addition of antigen to whole supernatants is unable to increase its inflammatory potency. In our view two possible mechanisms responsible for the formation of the skin reactive factor should be considered. The first possibility is that antigen interacts with a specific receptor on the sensitized lymphocyte surface resulting in the synthesis and or release of the inflammatory material from the cell itself. Similarly an interaction between antigen and an antibody-like material, possibly coating normal macrophages would result in the release of a mediator from the macrophages, possessing different characteristics. The second possibility is that exposure of sensitized cells to antigen results in stimulation of specific antibody synthesis or possibly of yet unassembled polypeptide chains possessing antigen combining sites. This results in the formation of soluble 'antigen–antibody' complexes, which are in turn responsible for the release of a non-specific inflammatory substance by normal lymphocytes and macrophages. The demonstration of γ-globulin synthesis in LN cultures and the finding of a possible PPD–antibody complex in LN supernatants gives additional strength to this second hypothesis.

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