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Demonstration of the Inflammatory Activity of the Supernatant of Hypersensitive Lymph Node Cells Incubated with a High Dose of Antigen

J. Krejčí, J. Pekárek, J. Johanovský and J. Švejcar

Research Institute of Immunology, Prague

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Summary. When lymphocytes from hypersensitive animals are incubated with antigen, biologically active substances are formed which inhibit the migration of mesenchymal cells from normal animals.

These substances were tested by intradermal injection in guinea-pigs and rabbits. The supernatants from incubation of lymphocytes with a high dose of antigen caused immediate pallor which lasted several hours. Later there was a macroscopic inflammation maximal at 24 hours. The histology was characteristic of a delayed hypersensitivity reaction.

The injection of the supernatant from hypersensitive lymphocytes incubated with a small dose of antigen caused little or no pallor and was not followed by a delayed inflammatory reaction. Injection of this supernatant together with the antigen did not potentiate or alter the reaction, in contrast to *in vitro* experiments where the inhibition of the migration by this supernatant was potentiated by antigen.

Besides this factor a distinct factor occurs in extracts and supernatant fluids of lymphocytes cultivated without antigen and those from control animals, which increases vascular permeability. This substance is probably identical with the lymph node permeability factor (LNPF). The possible role of these biologically active substances in the mechanism of delayed type hypersensitivity is discussed.

INTRODUCTION

During incubation of lymph node cells from sensitized animals with antigen, biologically active substances are released capable of inhibiting the migration of mesenchymal cells from normal non-sensitized animals (Bloom and Bennett, 1966; David, 1966; Švejcar, Johanovský and Pekárek, 1967).

The mechanism of action of these substances is not yet known. However, one component possessed an immunological specificity, and only inhibited migration after further addition of specific antigen (Švejcar *et al.*, 1967; Švejcar, Pekárek and Johanovský, 1968a; Bennett and Bloom, 1967). This immunologically specific component is produced on incubation of lymphoid cells with a small dose of antigen. If a higher dose of antigen is used, substances are formed and released which possess a direct migration inhibiting activity i.e. without further addition of antigen (Švejcar *et al.*, 1968b). So far the activity

of these substances has been mainly studied *in vitro* although Bennett and Bloom (1968) have investigated their participation in the *in vivo* manifestations of delayed hypersensitivity.

The lymph node permeability factor (LNPF) has been suggested as the pharmacological mediator of delayed hypersensitivity: see Willoughby, Boughton, Spector and Schild (1962), Willoughby, Spector and Boughton (1964) and Spector and Willoughby, (1964). Voisin and Toullet (1960) described permeability changes in delayed type hypersensitivity. Against this background we decided to test the substances released during *in vitro* incubation of hypersensitive cells with antigen by intradermal injection in the guinea-pigs, using the vascular permeability test and histological evaluation of the inflammatory reaction.

MATERIALS AND METHODS

Sensitization of rabbits

For the sensitization of rabbits heat killed *Mycobacterium tuberculosis* in adjuvant (Švejcar *et al.*, 1967) or four-times recrystallized ovalbumin prepared at our laboratory were used. The rabbits sensitized to ovalbumin were injected into both hind pads, with a dose of 100 μg ovalbumin in Freund's complete adjuvant (8·5 Bayol F, 1·5 Arlacel, 30 mg dried tubercle bacilli+3 mg ovalbumin and 5·0 ml physiological saline) in a dose of 0·5 ml/rabbit. The preparation of the nodes and of the active substances were carried out 10 days after the sensitization. The rabbits used as controls were pre-treated with incomplete adjuvant without antigen (Bayol F 8·5; Arlacel 1·5; physiological saline, 5·0 ml) (Švejcar *et al.*, 1968b).

Preparation of biologically active substances

Biologically active substances were obtained on incubation of lymph node cells from hypersensitive rabbits with antigen, using a technique described in our preceding communications (Švejcar *et al.*, 1967). Nomenclature of different supernatants: (1) supernatant A from incubation of cells without antigen; (2) supernatant B from incubation of cells with a high dose of antigen (100 μg PPD tuberculin or 1000 μg ovalbumin) and (3) supernatant C from incubation of cells with a low dose of antigen (1 μg PPD tuberculin or 50 μg ovalbumin).

We also studied cell extracts, since we found that active substances are produced within the cells at first and only later released into the medium (Švejcar *et al.*, 1967, 1968b). The supernatants and cell extracts were preserved by freeze drying immediately after their preparation. Their activity was controlled by the migration inhibition test (Švejcar *et al.*, 1967). Physiological saline served as diluent throughout these experiments.

Animals

Skin tests were performed in random bred albino guinea-pigs weighing 350 g of either sex, and in Chinchilla rabbits weighing 2·5–3 kg. A volume of 0·1 ml of supernatant was injected intradermally. Previous studies (Švejcar *et al.*, 1967) showed that it was possible to use one animal species for producing substances active in other species.

Determination of vascular permeability

This was performed by the Evans blue test (Krejčí, Pekárek and Johanovský, 1968).

Evaluation of pallor

Pallor was evaluated in a similar manner as the increased permeability, i.e. by calculating the surface area of the spots. Measurements were taken at 10, 20, 30, 40, 60, 90, 120, 180 and 240 minutes.

Histological examination

Pieces of skin were preserved with neutral formol, embedded in paraffin, and the sections stained with haematoxylin and erythrosin.

RESULTS

VASCULAR PERMEABILITY CHANGES IN GUINEA-PIG SKIN FOLLOWING INTRADERMAL INJECTION OF BIOLOGICALLY ACTIVE SUBSTANCES

In the first experiments cells sensitized to tubercle bacilli or ovalbumin were used. Supernatant A from incubation of hypersensitive rabbit lymph node cells without antigen was injected intradermally. Almost immediately after the injection a slight reddening developed around the site of injection; and the vascular permeability increased. This increase was maximal approximately 30 minutes after the injection and reached values about 30 mm^2. After 1 hour the vascular permeability decreased; at 6 hours and later it was the same as in controls. Macroscopically, no reactions were seen at 24 hours. There was no significant difference between the supernatant A and cell extract nor between the reaction elicited by the supernatant A obtained from rabbits sensitized to tuberculin or ovalbumin.

Supernatant B from incubation of hypersensitive cells with a high dose of PPD had an unexpected activity. Following intradermal injection there was considerable pallor and no increase of vascular permeability (Fig. 1a). Supernatant B was active even after dilution (see Table 1).

TABLE 1

THE ACTIVITY OF THE SUPERNATANT B (SUPERNATANT FROM INCUBATION OF LYMPH NODE CELLS FROM RABBITS SENSITIZED TO TUBERCULIN WITH A HIGH DOSE OF SPECIFIC ANTIGEN) IN GUINEA-PIG SKIN

Time	The mean area of pallor (mm^2) in skin tests				
	Undiluted solution	Dilution			
		1 : 5	1 : 10	1 : 50	1 : 100
10 minutes	353·6	138·0	152·4	70·0	61·0
20 minutes	463·4	223·6	228·8	88·6	25·2
30 minutes	454·8	200·0	225·0	135·0	19·8
40 minutes	450·0	180·4	180·6	90·8	0
60 minutes	385·0	143·6	124·6	16·2	0
90 minutes	358·2	98·4	54·0	0	0
120 minutes	250·4	24·0	0	0	0
180 minutes	122·4	0	0	0	0
240 minutes	57·6	0	0	0	0
24 hours*	68·2	22·0	15·6	17·8	13·8

* The 24-hour values represent the mean area of inflammatory skin reaction (induration and erythema) in mm^2.

The peak of the pallor appears after about 30 minutes. This is followed by gradual diminution, and after approximately 4 hours the pallor had disappeared. Its disappearance had a characteristic course. At first a very small flat reddening appeared in the centre and increased in size (Fig. 1b). The central reddening was accompanied by an increase of vascular permeability so that in 4–5 hours the pallor was replaced by erythema with increased vascular permeability. About 12 hours later the erythema was followed by induration. At 24 hours there was a considerable reaction which had the characteristic macroscopic appearance of a delayed hypersensitivity reaction.

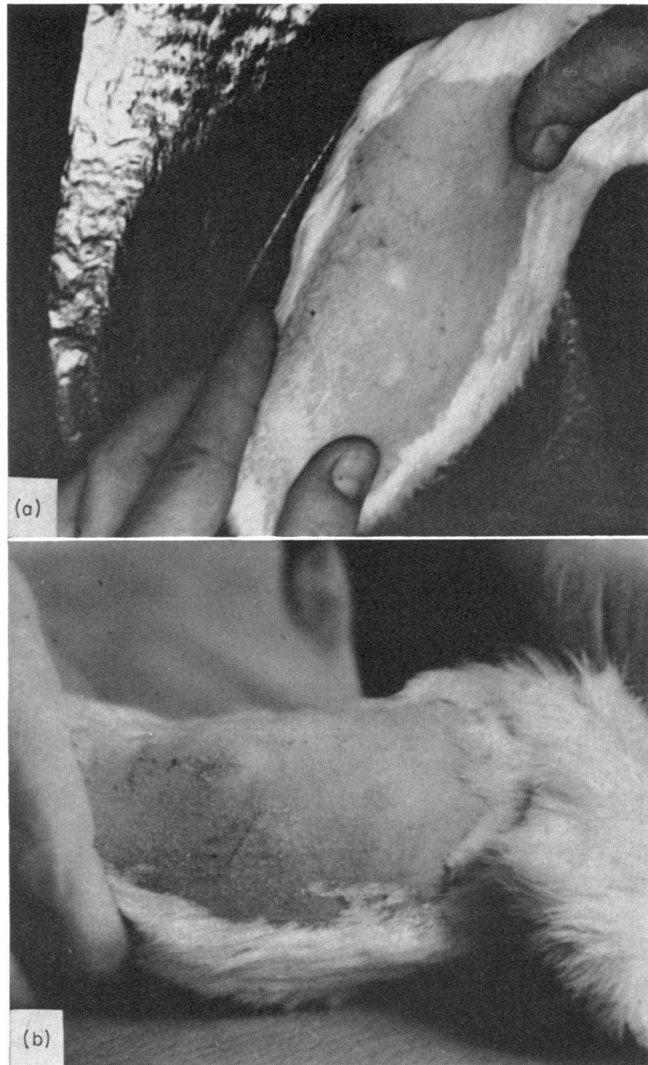

FIG. 1 (a) The pallor seen 15 minutes after injecting supernatant B into a normal guinea-pig. (b) The pallor and central erythema 3 hours after injecting supernatant B into a normal guinea-pig.

Similar reactions were observed with both the supernatant B of cell cultures and with cell extracts.

Supernatant fluid B obtained after incubation with the specific antigen of lymph node cells from rabbits sensitized to ovalbumin also caused pallor (see Table 2).

TABLE 2

THE ACTIVITY OF THE SUPERNATANT B (SUPERNATANT FROM INOCULATION OF LYMPH NODE CELLS FROM RABBITS SENSITIZED TO OVALBUMIN WITH A HIGH DOSE OF SPECIFIC ANTIGEN) IN GUINEA-PIG SKIN

Time	The mean area of pallor (mm^2) in skin tests					
	Undiluted solution	Dilution				Diluting solution
		1 : 5	1 : 10	1 : 50	1 : 100	
10 minutes	473·8	179·8	154·8	106·4	89·2	0
20 minutes	415·6	196·6	149·4	0	0	0
30 minutes	279·8	139·0	115·6	0	0	0
40 minutes	259·8	141·6	87·6	0	0	0
60 minutes	204·8	74·6	56·8	0	0	0
90 minutes	123·2	34·6	26·4	0	0	0
120 minutes	24·0	0	0	0	0	0
12 hours*	202·6	43·8	29·4	17·8	14·2	4·8
24 hours	242·4	56·0	25·0	9·4	7·8	5·0

* The 12- and 24-hour values represent the mean area of inflammatory skin reaction (induration and erythema) in mm^2.

Supernatant C released during the incubation of lymph node cells with a small dose of antigen caused slight pallor at first which soon changed into erythema which was associated with increased vascular permeability. The intensity and duration of the pallor corresponded approximately to a 1 : 50 dilution of the supernatant B.

It is interesting that further addition of antigen to the supernatant did not increase the intensity or change the character of the reaction. This is different from *in vitro* experiments in which the addition of antigen to supernatant C results in migration inhibition comparable with the inhibition obtained on using supernatant B.

Supernatant fluids and extracts of lymph node cells from normal non-sensitized rabbits cultivated with PPD of tuberculin were tested to confirm the immunological specificity of the processes leading to the formation of the biologically active substance capable of eliciting pallor of guinea-pig skin. Rabbits pre-treated with incomplete adjuvant without antigen were also used. Supernatants from this control system elicited only a moderate increase of vascular permeability in guinea-pigs in the 1st hour after injection, with a maximum in about 30 minutes. Pallor was never observed. These results are summarized in Table 3.

TABLE 3

SUMMARY OF ACTIVITIES OF SUPERNATANTS A, B AND C

Supernatants	Permeability	Pallor	Inflammation at 24 hours	Inhibition of cell migration
A	+	−	−	−
B	−	+	+	+
C	+	−	−	−
C+antigen	+	−	−	+
Control supernatants	+	−	−	−

*See Švejcar *et al.* (1967, 1968a, b).

HISTOLOGICAL EXAMINATION OF SKIN TESTS PERFORMED WITH BIOLOGICALLY ACTIVE SUBSTANCES

Skin tests were performed by injecting 0·1 ml of the supernatants A, B or C into depilated skin of guinea-pigs or rabbits. Samples for histological examination were taken after 3, 12 and 24 hours. Following the administration of biologically active supernatants (A, B, C and C + antigen) a slight inflammatory infiltration developed in the subcutis in the first 3 hours after the injection, consisting mainly of polymorphonuclears. Leucostasis, and in some preparations a slight leukodiapedesis, mainly from dermal venules, was found.

Only supernatant B caused necrosis of isolated subcutaneous muscle fibres. This necrosis was characterized by swollen and homogenized structure of muscle fibres without any local cellular infiltration.

Twelve and 24 hours after the injection of supernatants A, C and C+antigen the histology was virtually negative. There were a few degenerating polymorphonuclears or a slight oedema of the subcutaneous connective tissue. A positive finding, however, was obtained after injecting supernatant B, where in most cases a typical inflammatory infiltration was found consisting of polymorphonuclear and mononuclear cells; in more than half the cases lipophagic foci and necrosis of single muscle fibres were found. Necrotic muscle fibres were often degraded by macrophages (Fig. 2). Similar findings were obtained in experiments performed with either supernatants or extracts obtained on incubation with the respective antigen of lymph node cells from rabbits sensitized to tuberculin or ovalbumin.

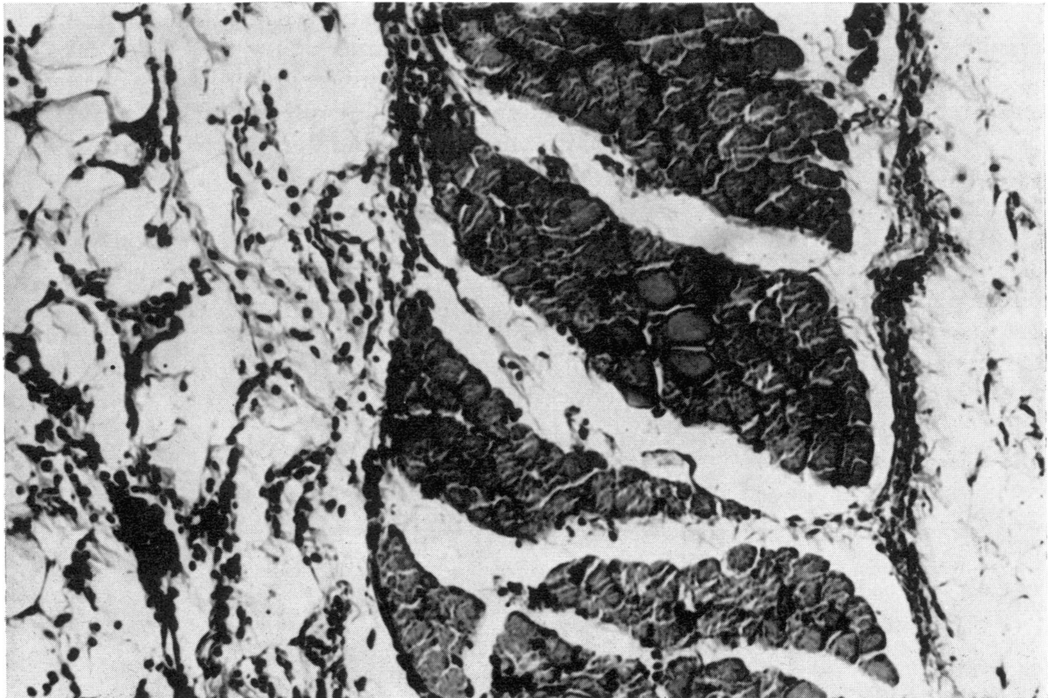

FIG. 2. Histological picture of an inflammatory reaction 24 hours after the intradermal injection of supernatant B into the skin of a normal guinea-pig. H & E, × 150.

DISCUSSION

The aim of the present study was to test for the *in vivo* activities of the supernatants produced by incubation of hypersensitive lymph node cells with the antigen. These supernatants are known to contain migration inhibiting factor (Bloom and Bennett, 1966; David, 1966; Švejcar *et al.*, 1967).

We found that supernatants from sensitized lymph node cells incubated alone or with high or low doses of antigen had distinctive effects when injected intradermally.

The following conclusions may be drawn:

(1) B supernatant produced by incubation of hypersensitive lymph node cells with a large dose of antigen causes pallor and produces lesions resembling a delayed hypersensitivity reaction *in vivo* when injected intradermally. It also inhibits cell migration *in vitro*.

(2) The substance is different from LNPF, because it has a reverse effect on vascular permeability when administered intradermally and was not found in other experimental or control conditions in which a substance resembling LNPF was obtained.

(3) There is a difference between the results obtained *in vivo* and *in vitro*. *In vivo*, supernatant B (cells incubated with a high dose of antigen) causes pallor and cell infiltration while supernatant C (cells incubated with a low dose of antigen) is much less active and its activity is not increased by adding antigen. Supernatant C is also less active than supernatant B in causing inhibition of migration of peritoneal exudate cells *in vitro* but supernatant C becomes as active as supernatant B on adding antigen.

The results indicate that there are probably at least three biologically active substances formed and released during incubation of hypersensitive lymph node cells with the antigen *in vitro*: pharmacological substances demonstrable by skin test or cell migration inhibition; an antibody-like substance demonstrable by inhibition of migration when further antigen dose is added to the test system; and a substance resembling the lymph node permeability factor.

Preliminary experiments suggest that the first substance is not adrenaline, 5-hydroxytryptamine or endotoxin. It may be related to the agents responsible for lymphocyte-mediated cytotoxic phenomena *in vitro* (Rosenau, 1968; Holm and Perlmann, 1967; Ruddle and Waksman, 1967). The second, antigen-dependent, active substance may be correlated to some of the recently described phenomena of passive *in vitro* transfer of delayed type hypersensitivity (Fireman, Boesman, Haddad and Gitlin, 1967; Thor, 1967) and the behaviour of cytophilic antibodies (Nelson and Boyden, 1967).

Our results on LNPF are not quite identical with those of Spector and Willoughby (1964); the doses used in our experiments were, however, substantially lower. A distinct problem is the participation of LNPF in later, secondary, stages of development of delayed type hypersensitivity or other inflammatory reactions.

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