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Production of Pyrogenic Substances in the Reaction of Cells of Hypersensitive Guinea Pigs with Antigen *in vitro*

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Summary. Previous findings of the appearance of pyrogenic activity when spleen and lymph node cells from BCG-sensitized rabbits were incubated with tuberculin *in vitro* have been repeated and extended in experiments with guinea pigs. Two different systems were used: hypersensitivity induced by BCG infection and by means of diphtheria toxoid-antitoxin precipitates. Cells were incubated *in vitro* with diluted tuberculin, diphtheria toxoid or Hanks's solution. Supernatant fluids obtained after various incubation periods were tested in normal guinea pigs for their pyrogenic activity and for ability to induce skin inflammatory reactions.

Pyrogenic substances were obtained in most of the experiments in which cells of hypersensitive animals were incubated with the specific antigen; experiments with the unrelated antigen and controls were negative. Formation of skin-irritating substances was less regular, and they were sometimes formed non-specifically in control experiments also; in general, however, the results of these experiments agreed with the results of the fever reaction.

The results obtained are discussed in relation to the mechanism of delayed hypersensitivity.

INTRODUCTION

HYPERSENSITIVITY of the delayed type has three principle manifestations: an inflammatory skin reaction, systemic pyrexial reaction and changes (mostly injurious) of hypersensitive cells on contact with antigen *in vitro*. Passive transfer experiments reveal that the inflammatory skin reaction in delayed hypersensitivity is due to the direct reaction of hypersensitive cells with antigen (Metaxas and Metaxas-Buehler, 1955; Inderbitzin, 1956; Waksman and Matoltsy, 1958). It may be supposed that in the reaction of such hypersensitive cells with antigen *in vivo* substances (intermediate products) with biological activity are formed which play a part in provoking the allergic manifestations (Rich, 1951; Boyden, 1958; Wesslen, 1952; Waksman, 1958). Arguments in favour of this hypothesis have been put forward in the case of inflammatory skin reaction by Berdel and Buddecke (1950) and Carrère and Quatrefages (1952), and in the case of the systemic tuberculin reaction by Johanovský (1959) and Hall and Atkins (1959) as a result of their demonstration of the production of a form of endogenous pyrogen.

Attempts have been made in the past, by incubation of hypersensitive cells with the corresponding antigen *in vitro*, to obtain substances which would cause a reaction of the tuberculin type on intradermal injection into normal animals (Zinsser and Tamiya, 1926; Balteanu, Tom and Garaguli, 1938; Carrère and Quatrefages, 1952), occasional positive results were not very convincing. Waksman and Matoltsy (1958) in other experiments found that the results varied according to whether hypersensitive cells were destroyed before or after incubation with tuberculin *in vitro*; in the latter case, skin reactions elicited in normal recipients were slightly stronger.

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It has already been reported (Johanovský, 1958, 1959) that during incubation *in vitro* with dilute tuberculin of cells from rabbits sensitized to BCG (white blood cells, spleen cells, and pure lymphocytes from cisterna chyli), substances were produced capable of provoking immediate short monophasic fever followed by lymphopenia on intravenous injection into normal recipients; after intradermal injection an inflammatory reaction appeared in 24 hours. No such substances were detected in control tests. The same experimental procedure, that is, incubation of hypersensitive cells with the corresponding antigen *in vitro* followed by assay of the biological activity of the supernatant fluids has been used in experiments with guinea pigs sensitized either by BCG vaccination or by diphtheria toxoid-antitoxin precipitates by the method of Uhr, Salvin and Pappenheimer (1957). The results presented show that in both cases it is possible by a specific reaction of hypersensitive cells with antigen *in vitro*, to obtain substances which imitate the manifestations of the systemic as well as local delayed hypersensitivity reaction.

MATERIAL AND METHODS

Laboratory animals

Guinea pigs used for sensitization were of both sexes, weighing 400–600 g., mostly albinos. As recipients (test animals) guinea pigs weighing 300–400 g. were used; for skin tests only albino guinea pigs were selected.

Sensitization

Guinea pigs were injected intraperitoneally with different doses (2–10 mg.) of live BCG. After 3–4 weeks, skin tests were carried out with 10, 2 and 0.4 μg. of purified tuberculin. Animals which developed a satisfactory degree of skin hypersensitivity were selected for further experiments. Guinea pigs sensitized to diphtheria toxoid received four doses of 0.1 ml. of diphtheria toxoid-antitoxin precipitate (in incomplete adjuvants without Mycobacteria) into the foot-pads of all four extremities on the same day. The injected material contained 0.5 ml. light mineral oil, 0.2 ml. lanolin, and 0.3 ml. diphtheria toxoid-antitoxin precipitate in 1 ml., the content of toxoid being 4 Lf; the immunization dose for each animal amounted to approximately 0.5 Lf. Guinea pigs in individual experiments were tested at various intervals (usually 7–10 days) after sensitization by intracutaneous injections of 1 Lf diphtheria toxoid; skin reactions were read 4, 24 and 48 hours later. After the last reading, or on the following day, animals were used for experiments. In some experiments guinea pigs were tested for manifestations of systemic hypersensitivity by intracardiac injection of 1 Lf diphtheria toxoid or 5 μg. purified tuberculin. The doses were less than $\frac{1}{5}$ of the minimum dose pyrogenic for normal guinea pigs.

Antigens

Tuberculin used in most of our experiments was partially purified, prepared from non-autoclaved filtrates of sterilized cultures of Mycobacterium tuberculosis H 37 RV by the Pankborn-Birkhang technique (1954) at the Institute of Tuberculosis Research, Prague (Kára, 1956). In a part of the experiments purified tuberculin (PPD) from Statens Seruminstitut, Copenhagen, was used. The results attained were the same with both preparations.

Purified diphtheria toxoid was prepared at the Institute of Sera and Vaccines, Prague, and contained 2800 Lf/ml. As antitoxin for the preparation of precipitates diphtheria

flocculating horse antiserum was used, containing 950 Lf/ml. Precipitates were prepared according to Uhr, Salvin and Pappenheimer (1957) in the zone of slight antibody excess (5–10 per cent), and were washed three times in buffered phosphate saline at pH 7.2.

Cells

In experiments with BCG sensitized guinea pigs, isolated spleen cells and mesenteric lymph node cells were used; in experiments with animals sensitized by diphtheria precipitates we used spleen cells and axillary, subscapular, popliteal, femoral and inguineal lymph node cells. The animals were bled out from the carotid artery and the spleen and lymph nodes were removed under sterile conditions. The cells were further treated either in a refrigerating box at 4° or in vessels filled with ice. Spleens and lymph nodes were minced in sterile Petri dishes and the contents carefully squeezed out and ground with forceps and a fine scalpel (Šterzl, 1957). The suspension thus obtained was further stirred in a small quantitity of Hanks's solution and the resulting dense cell suspension repeatedly sucked up and squirted out with a pipette or syringe. After allowing the tissue fragments to settle a suspension was obtained consisting of individual cells with a few small clusters of 3–10 cells. The cells obtained were washed four times on the centrifuge in the cold at 375 *g* for 5–7 minutes in 6–8 ml. of Hanks's solution.

Experimental procedure

The washed cells from 5–8 donors were suspended in several test tubes in 2–4 ml. of Hanks's solution containing penicillin (100 units/ml.) and streptomycin (100 μg./ml.). In the majority of experiments, one tube was left as a control and to another was added 0.2 ml. diluted purified tuberculin (2 μg.), and to a third 0.2 ml. (0.5 Lf) diluted diphtheria toxoid. The quantity of cells in single test tubes varied in individual experiments between 50–300 million. The proportion of viable cells was 70–80 per cent for spleen cells, and 80–90 per cent for lymph node cells, when the viability was determined by suspending in 1 per cent Congo red solution and observing the number of unstained cells in fresh preparations at magnification ×450.

Cell suspensions were incubated in a thermostat at 37°. At various time intervals the cells were centrifuged, the supernatant fluid preserved, and the cells were resuspended in Hanks's solution containing antigen and reincubated. In some experiments the supernatant fluids were taken after 2, 4 and 20 hours' incubation, in others after 3–4 and 20 hours' incubation only; in a few experiments the cells were centrifuged and the supernatant fluid collected after 1 hour incubation. The supernatant fluids were tested for sterility and stored in frozen state (−20°) until further use.

Testing of samples

The capacity of supernatant fluids to induce fever was tested in normal guinea pigs by intracardiac injections. The injections were made very slowly, and during and at the end of the injection the correct position of the needle in the heart was ascertained by sucking up blood. Intracardiac injection of non-pyrogenic fluids never caused a rise in temperature in any of the numerous control guinea pigs. Sometimes a temporary fall of 0.2–0.4° occurred, lasting mostly ¼ or ½ hour. Rectal temperatures were taken with a thin mercury thermometer, twice during the ½ hour immediately before the experiment, and ½, 1, 2, 3, 4 and 6 hours after injection of the test fluid. Guinea pigs whose temperature before the experiment was higher than 39.5° or in which there was a difference between the pre-

liminary readings exceeding 0.2° were excluded. Guinea pigs were used for testing once or twice a week at the most, and after three tests they were excluded. The capacity of supernatant fluids to cause inflammation was tested by intracutaneous injections of 0.1 ml. into shaved and depilated normal albino guinea pigs; reactions were read after 8, 24, 48 and 72 hours.

Control measures

Measures were taken to prevent contamination by pyrogenic material. All solutions and other material used were repeatedly tested for sterility and absence of pyrogens. Laboratory glassware, test tubes, pipettes, etc., were sterilized by hot air at 170°; syringes and needles either in the same way or, after sterilization by boiling, washed repeatedly in sterile, pyrogen-free saline.

The design of each experiment, in which cells were incubated under similar conditions with a specific antigen, a non-specific antigen and without antigen, provided a further check on the reliability of the procedures.

RESULTS

In all guinea pigs sensitized by intraperitoneal injections of live BCG vaccine there developed in 3–4 weeks a skin hypersensitivity characterized by erythema and induration (15–20 mm.) after injecting 2 and 0.4 μg. purified tuberculin. Similar inflammatory reactions appeared in three-quarters of the guinea pigs sensitized by diphtheria toxoid-antitoxin precipitates when tested with 1 Lf diphtheria toxoid. Reactions in the remaining animals were weak or negative. In both groups of animals the reactions were of the same type, detectable macroscopically at 24 hours, and lasting in the case of tuberculin reactions up to 72 hours and, in the case of diphtheria toxoid reactions, up to 48 hours. Such reactions are characteristic of delayed-type hypersensitivity. Skin hypersensitivity appeared in guinea pigs sensitized by diphtheria toxoid in 7–14 days after injection of precipitates, with insignificant variations in individual experiments; after this period it usually decreased. In the course of 2 weeks no antibodies (haemagglutinins) were detectable in the sera of these animals, while 3 weeks after sensitization they could be demonstrated in rare cases only and in low titres, from 1/2 to 1/16

Guinea pigs sensitized with BCG vaccine were used for experiment usually 3–10 days after intradermal testing, animals sensitized with diphtheria precipitates almost always 2 days after the skin test. A number of guinea pigs from each group were tested 2 days after the intradermal test by intracardiac injection of the respective antigens. In both groups the hypersensitivity reaction was practically the same, fever beginning after a latent period of ½–2 hours, lasting a few hours and reaching 1° or more; a characteristic biphasic type of fever was recorded in certain cases (Fig. 1). An approximate relation was found between the intensity of skin hypersensitivity and the degree of temperature increase in systemic reactions.

Cells of hypersensitive guinea pigs (spleen and lymph node cells) were mixed *in vitro* with diluted diphtheria toxoid or tuberculin, or Hanks's solution, incubated at 37°, and centrifuged at various intervals; supernatant fluids were tested for their pyrogenic activity in normal guinea pigs. Some typical results are given in Fig. 2. In contrast to the systemic reaction provoked in actively sensitized guinea pigs, there occurs a short monophasic temperature increase with or without a short latent period. In test animals treated with

control supernatant fluid (obtained by incubation of cells without antigen or with the non-specific antigen) a short-term temperature decrease was noted in most cases, most probably due to the effect of the intracardiac injection. This reaction could to a certain extent interfere with the specific pyrogenic activity of the supernatant fluid. In rare cases

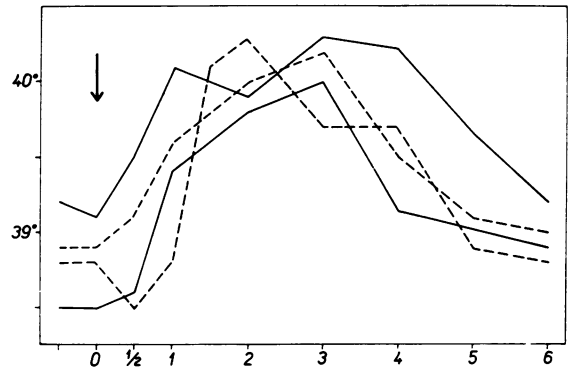

FIG. 1. Examples of systemic delayed hypersensitivity reaction.
Abscissa: time in hours
Ordinate: temperature in degrees
Full line: 5 µg. PPD to BCG-sensitized guinea pigs
Dashed line: 1 Lf diphtheria toxoid to guinea pigs sensitized by diphtheria precipitates

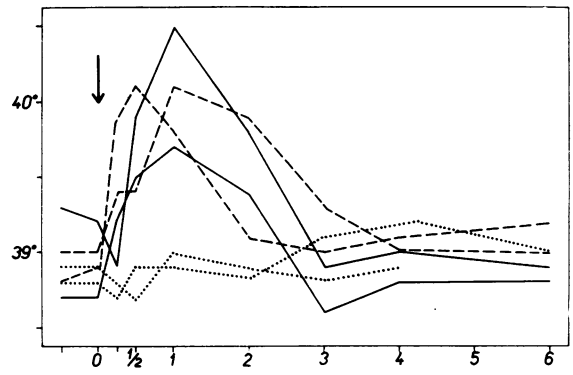

FIG. 2. Examples of reaction of test guinea pigs to intracardial injection of supernatant fluids obtained by incubation of hypersensitive cells *in vitro*.
Abscissa: time in hours
Ordinate: temperature in degrees
Full line: cells of BCG-sensitized guinea pigs + 2 µg. PPD
Dashed line: cells of guinea pigs sensitized to diphtheria toxoid + 0.5 Lf toxoid
Dotted line: cells of the same type + non-specific antigen

a mild progressive temperature increase was noticed 3 or more hours after the injection of the test fluid. This was probably a non-specific response to the irritation induced by injection and was not considered as a positive fever reaction.

Positive results were obtained in both types of hypersensitivity when cells were mixed with the corresponding antigen; incubation of hypersensitive cells with the unrelated

antigen did not result in the production of pyrogenic substances. The experiments were carried out in a total of 277 guinea pigs. The difference between the temperature before the experiment and that attained during the first hour after the injection of supernatant fluid was used for evaluating the degree of the temperature reaction. All results are summarized in Fig. 3. Supernatant fluids obtained by incubation of cells from hypersensitive guinea pigs with the respective antigen usually induced fever reactions of varying intensity, whereas the supernatant fluids obtained by incubating the same cells alone or with the unrelated antigen were inactive. For purposes of comparison temperature increase lower than 0.4° was regarded as negative, 0.5–0.9° as slightly positive and 1° or more as positive fever reaction.

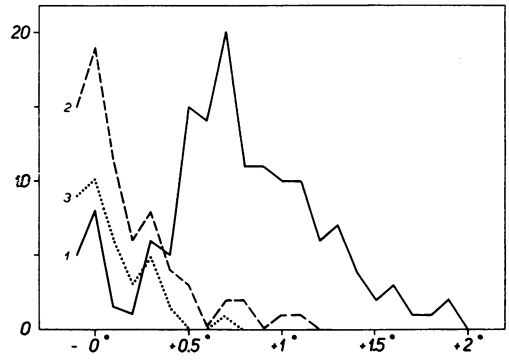

FIG. 3. Summary of assays of pyrogenic activity of supernatant fluid from cells of guinea pigs sensitized with BCG and diphtheria toxoid.
Abscissa: temperature change in degrees
Ordinate: number of experiments with results specified
Full line (1): cells+specific antigen
Dashed line (2): cells+non-specific antigen
Dotted line (3): cells+Hanks's solution

In a number of experiments the quantitative characteristics of the formation of pyrogenic substances by hypersensitive cells *in vitro* were studied. A greater and earlier production of pyrogenic substances was found not only when the number of hypersensitive cells was larger but also when more of the antigen was used (Table 1).

Results with various experimental groups of BCG-sensitized guinea pigs (various types of cells and times of incubation) are presented in Table 2, and with guinea pigs sensitized to diphtheria toxoid in Table 3. In the different groups the percentage of slightly positive and positive results was about the same. In the group in which cells were incubated for 3–4 hours are included some experiments in which the pyrogenic supernatant fluid was obtained after 2 and sometimes even 1 hour's incubation only. Evidence for the specificity of production of pyrogenic substances during incubation of hypersensitive cells with antigen *in vitro* is summarized in Table 4.

The amounts of antigen used for the production of pyrogenic substances by hypersensitive cells *in vitro* were usually 2 μg. PPD and 0.5 Lf diphtheria toxoid, but in some experiments even less. Non-specific pyrogenic reactions occurred in normal guinea pigs only when the amounts used were as much as 10 Lf diphtheria toxoid or 20 μg. PPD. It is reasonable to conclude, therefore, that the fever provoked after the injection of supernatant fluids in test guinea pigs could not have been due to the pyrogenic effect of

TABLE 1

EXAMPLE OF AN EXPERIMENT ON THE FORMATION OF PYROGENIC ACTIVITY DURING INCUBATION OF HYPERSENSITIVE CELLS (SENSITIZED TO DIPHTHERIA TOXOID) WITH ANTIGEN 'IN VITRO'

Antigen	*Number of cells*	*Incubation time (hours)*	*Temperature changes in recipients*	
			Supernatants of Spleen cells	*Supernatants of Lymph node cells*
Tuberculin 2 μg.	200×10^6	1.5	−0.3°	—
		3	−0.1°	+0.1°
		20	+0.1°	−0.2°
Diphtheria toxoid 0.5 Lf	300×10^6	1.5	+0.7°	+0.9°
		3	+1.1°	+1.7°
		20	+1.3°	+1.2°
	100×10^6	1.5	+0.3°	+0.4°
		3	−0.2°	+1.2°
		20	+1.1°	+1.3°
Diphtheria toxoid 0.1 Lf	200×10^6	1.5	+0.2°	−0.3°
		3	+0.5°	+0.8°
		20	+0.9°	+1.1°

TABLE 2

CAPACITY OF SUPERNATANT FLUIDS FROM CELLS OF BCG-SENSITIZED GUINEA PIGS TO PRODUCE PYROGENIC ACTIVITY ON INCUBATION WITH TUBERCULIN (PPD 2 μG. OR LESS), DIPHTHERIA TOXOID (0.5 OR 1 LF), OR HANKS'S SOLUTION 'IN VITRO'

Incubation fluid	*Incubation time (hours)*	*No. of recipients showing temperature changes*					
		Spleen cells			*Lymph node cells*		
		< 0.4°	0.5–0.9°	> 1°	< 0.4°	0.5–0.9°	> 1°
Tuberculin	3–4	4	11	7	1	7	5
	20	3	9	6	2	7	4
Diphtheria toxoid	3–4	8	—	—	6	—	—
	20	8	1	1	7	—	—
Hanks's solution	20	18	1	—	15	—	—

TABLE 3

CAPACITY OF SUPERNATANT FLUIDS OF CELLS OF GUINEA PIGS SENSITIZED BY DIPHTHERIA TOXOID-ANTITOXIN PRECIPITATES TO PRODUCE PYROGENIC ACTIVITY ON INCUBATION WITH DIPHTHERIA TOXOID (0.5 LF OR LESS), TUBERCULIN (PPD 2 μG.), OR HANK'S SOLUTION

Incubation fluid	*Time (hours)*	*No. of recipients showing temperature changes*					
		Spleen cells			*Lymph node cells*		
		< 0.4°	0.5–0.9°	> 1°	< 0.4°	0.5–0.9°	> 1°
Diphtheria toxoid	3–4	5	5	5	4	13	5
	20	—	8	8	4	8	6
Tuberculin	3–4	12	1	—	11	2	1
	20	9	—	1	10	2	—
Hanks's solution	20	7	—	—	19	—	—

the antigens used. Moreover, in all experiments both antigens were applied in parallel, and each group acted as a control for the other.

In most experiments 0.1 ml. of the supernatant fluids were also tested intracutaneously — i.e. in a dose approximately $\frac{1}{20}$ the amount tested for its capacity to cause fever. The

TABLE 4

SPECIFICITY OF PRODUCTION OF PYROGENIC ACTIVITY BY CELLS OF HYPERSENSITIVE GUINEA PIGS IN CONTACT WITH ANTIGEN 'IN VITRO'

Incubation in vitro	*Temperature changes in recipients*					
	BCG-sensitized guinea pigs			*Diphtheria toxoid-sensitized guinea pigs*		
	< 0.4°	0.5–0.9°	> 1°	< 0.4°	0.5–0.9°	> 1°
Tuberculin	10	34	22	42	5	2
Diphtheria toxoid	29	1	1	13	34	24
Hanks's solution	33	1	—	26	—	—

TABLE 5

CORRELATION BETWEEN THE INTENSITY OF FEBRILE REACTION AND SKIN INFLAMMATORY REACTION EVOKED BY SUPERNATANT FLUIDS OBTAINED BY INCUBATION OF HYPERSENSITIVE CELLS WITH ANTIGEN 'IN VITRO'

Rise of temperature	*No. showing inflammatory reactions (diameter in millimetres)*			
	10–15	5–10	< 5	0
1° or more	12	9	5	—
0.5–0.9°	12	18	12	6
< 0.4°	5	10	19	23

TABLE 6

NUMBER OF INFLAMMATORY REACTIONS OF VARYING INTENSITIES EVOKED BY SUPERNATANT FLUIDS OBTAINED BY INCUBATING HYPERSENSITIVE CELLS WITH SPECIFIC AND NON-SPECIFIC ANTIGEN 'IN VITRO'

Cells of BCG sensitized guinea pigs incubated with:	*No. of recipients with inflammatory reactions (diameter in millimetres)*			
	10–15	5–10	< 5	0
Tuberculin	17	9	11	4
Toxoid	3	5	9	8
Cells of guinea pigs sensitized by diphtheria toxoid incubated with:				
Tuberculin	2	3	8	14
Toxoid	9	20	7	3

results of readings of inflammatory reactions after 24 and 48 hours are given in Tables 5 and 6. Comparison of the ability of the individual supernatant fluids to cause pyrogenic and inflammatory reactions (Table 5), or of the occurrence of inflammatory reactions with supernatant fluids obtained after incubation with specific and non-specific antigens

(Table 6) reveals some positive correlation, but also a considerable proportion of incongruous results. The difference between the control and experimental groups is most marked when those animals with the most intense and those with negative reactions are compared.

DISCUSSION

The results show a close correlation between the state of delayed hypersensitivity in guinea pigs and the ability of cells from these animals to release pyrogenic substances when incubated with the corresponding antigen *in vitro*, and are in agreement with our previous results in rabbits (Johanovský, 1958, 1959). The similarity of the findings with cells of BCG-sensitized guinea pigs in contact with tuberculin and with cells of guinea pigs sensitized by diphtheria precipitates in contact with diphtheria toxoid indicates that in both cases an identical type of reaction occurs. This indicates that the hypersensitivity provoked by diphtheria toxoid can properly be considered as delayed type hypersensitivity (Uhr *et al.*, 1957; Raffel and Newel, 1958).

Taken all together, our findings show that the reaction which occurs is immunologically specific. It is impossible to decide whether occasional positive results in the control groups should be interpreted as due to contamination by exogenous pyrogenic substances, etc., or whether they indicate some degree of non-specificity of the reaction. It is known that delayed hypersensitive animals show increased susceptibility to the pyrogenic or lethal activity of bacterial endotoxins (Abernathy, Bradley and Spink, 1958; Howard, Biozzi, Halpern, Stiffel and Mouton, 1959; Stetson, Schlossman and Benacerraf, 1958; Suter, Ullman and Hoffman, 1958; Uhr and Brandriss, 1958).

There is a general correlation between the existence or absence of the pyrogenic action and of the skin inflammatory reaction caused by supernatant fluids; a partial correlation can also be found between the intensity of both phenomena. This relationship holds no more and no less than that between the intensity of skin and systemic hypersensitivity reactions in actively sensitized guinea pigs. It is necessary to take into account the fact that the macroscopic evaluation of weak inflammatory reactions is rather difficult and that the intensity of inflammatory reactions may be affected by a number of products of cellular disintegration as well as by other non-specific factors. These problems have been discussed in detail in a recent paper (Johanovský, 1959).

Experiments of other workers concerning the appearance of skin-irritating substances during incubation of cells from hypersensitized animals with antigen *in vitro* were quoted in the introduction of this report. Our experiments have confirmed the general conclusion that such a phenomenon takes place, although a number of irregularities and exceptions were observed. The fundamental difference between the systemic (pyrogenic) and the delayed skin-hypersensitivity reactions seems to lie in the fact that in the pyrogenic reaction the substances released from injured cells travel via the circulation directly to the thermoregulatory centres to provoke a febrile reaction (Hall and Atkins, 1959). In the skin tuberculin reaction on the other hand, a whole sequence of cellular events is required to give rise to typical inflammatory manifestations. This sequence of events is probably never completely imitated by a simple injection of the substances produced *in vitro*. For these reasons the systemic tuberculin reaction is probably more suitable than the skin test for the demonstration of irritating substances formed *in vitro*. This may explain the difference in outcome of the two types of experiment.

The demonstration of skin-irritating activity in the supernatant fluids obtained by

incubation of hypersensitive cells *in vitro* may be taken to support the basic importance of the demonstration of pyrogenic activity. Since it is possible, by eliciting a systemic reaction, to bring about skin desensitization at the same time (Uhr and Brandriss, 1958) we may suppose that substances arising in our *in vitro* experiments are related to the phenomena of systemic as well as of skin hypersensitivity.

It is not possible at present to relate the observations described above to the experiments of Lawrence and Pappenheimer (1957) on the release of the so-called 'transfer factor' by antigen from hypersensitive cells in man. The basic question is how far the production of pyrogenic and skin-irritating substances by hypersensitive cells mixed with antigen *in vitro* can be considered to reflect the events occurring when delayed hypersensitivity reactions take place *in vivo*. In other words, whether the activities observed (which seem capable of imitating the phenomena of delayed hypersensitivity *in vivo*) are actually due to the same substances or mechanisms as mediate these manifestations under normal *in vivo* conditions, or whether they are merely a consequence of injury to hypersensitive cells by antigen *in vitro*, without any causal relation to the phenomena of hypersensitivity *in vivo*.

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