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Radiometric ear index test as a measure of delayed-type hypersensitivity in the rat

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Summary. Intradermal skin tests performed in the pinna of the rat ear appeared to be 100 times more sensitive than classical flank skin tests in measuring Arthus and delayed-type hypersensitivity (DTH) reactions. One of these tests was antigen-induced thickening of the pinna of the ear. It was found to be a sensitive measure of Arthus reactivity at 4 h after irritation with antigen in both actively immunized rats and recipients of precipitating immune serum. The other test, radiometric ear index determination, exploits the fact that monocytes and monocyte derived macrophages accumulate at DTH reaction sites. The test was performed by labelling the precursors of these cells with a pulse of [^{3}H]-thymidine and by determining radioactivity in biopsy specimens taken from test sites in the pinna of the ear. At a certain antigen dose range this objective and highly sensitive method was shown to measure a purely cell mediated reaction which could be transferred to normal recipients with thoracic duct lymphocytes but not with immune serum. It also behaved as a typical DTH reaction in response to desensitizing injections of the specific antigen. Testing with unnecessarily high antigen doses, however, should be avoided since the strong early inflammation induced by them may interfere with the determination of DTH while using this sensitive assay.

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INTRODUCTION

Appropriately sensitized rats develop a classical delayed-type hypersensitivity (DTH) reaction in response to an intracutaneous injection of the specific antigen (Flax & Waksman, 1962). These reactions produce an induration which can be measured by palpation or by determining increase in skin thickness using special instruments. In order to achieve consistent results, however, the measurements should be done by one experienced person throughout the study. The erythema component of the DTH reaction in the rat is often pale which leads to special difficulties when using dark-skinned rat strains.

A number of more objective methods to measure DTH have been described (Axelrad, 1968; Sabolovic *et al.*, 1972; Lefford, 1974; Vadas, Miller, Gamble & Whitelaw, 1975; Franco & Morley, 1976). One of these, the technique used by Lefford (1974) exploits the fact that radioactively labelled blood monocytes and monocyte-derived macrophages accumulate in substantial numbers at DTH reaction sites in the pinna of the rat ear. This procedure has been used in studies of infection immunity to measure DTH induced by bacteria such as BCG (Lefford, 1974), *Francisella tularensis* (Kostiala, McGregor & Logie, 1975) and *Listeria monocytogenes* (Kostiala & McGregor, 1975) as well as to ovalbumin attached to BCG (Crum & McGregor, 1976).

The results of the present study show that this radiometric ear index test is highly specific and

extremely sensitive in classical DTH induced by protein antigens in Freund's complete adjuvant (FCA). It will also be shown that antigen induced induration at a skin test site can be measured much more sensitively in the pinna of the rat ear than in the flank. Provided that the antigen test dose is low, precipitating antibody will be excluded as a mediator of reactions measured in the radiometric ear index test. This assay will be shown to measure DTH that can be transferred to normal recipients with thoracic duct lymphocytes (TDL). Both serum and cell mediated reactions, however, can be measured in a sensitive way by determining the increase in ear thickness.

MATERIALS AND METHODS

Animals

Inbred male and female Sprague-Dawley rats were used. The success of inbreeding has been confirmed by transfer of skin grafts which have always survived up to 12 months without signs of rejection. At the beginning of the experiments the rats were 8–10 weeks old and weighed 150–250 g. No precautions were undertaken to maintain them in specific pathogen free conditions. They were kept in roomy cages and fed commercial rat pellets, oat grain, bread and water *ad libitum*.

Immunization

Rats were immunized with 500 μg of bovine serum albumin (BSA, Sigma Chemical Co, St. Louis, Mo.) or 240 μg of purified diphtheria toxoid (DT, Orion Pharmaceutical Co., Helsinki) into a hind footpad. Another group of rats was sensitized with both of these proteins intradermally in the neck. The proteins were emulsified 1:1 in Freund's complete adjuvant (FCA) consisting of 85% of Bayol 55 (Esso), 15% of Arlacel A (mannide monooleate, Atlas Powder Co., Wilmington, Delaware) and 6 mg/ml of heat killed *Mycobacterium tuberculosis* (C, DT and PN strains, Massachusetts General Hospital, Boston). The adjuvant mixtures were injected in volumes of 0·1 ml.

Flank skin test

Intradermal tests were performed in the right flank with 50, 5 or 0·5 μg of BSA in 0·1 ml of 0·85% saline. The results were recorded at 4, 24 and 48 h by measuring the diameter of erythema. A value greater than 10 mm was defined as a positive reaction. In addition, the intensity of induration was simultaneously determined with a caliper (Oditest, H. C. Kröplin, Schluectern) as an increase in skin thickness from the value measured immediately before intradermal injection.

Radiometric ear index test

The technique used in the present study was modelled on the procedure described by Lefford (1974). The rats were given a subcutaneous pulse of 0·25 μCi/g body weight of [^{3}H]-thymidine (5 Ci/mM, Amersham, England). Twenty four hours later either BSA in amounts of 50, 5, 0·5, 0·05, or 0·005 μg or DT in amounts of 24 or 0·24 μg diluted in 20 μl of 0·85% saline was injected into the central part of the pinna of the right ear using 50 μl Hamilton syringes with 27 G needles. Similarly, 20 μl of saline diluent were injected into the pinna of the left ear. After 4, 24 and 48 h, groups of rats were killed and uniform pieces of tissue 6 mm in diameter were removed from the injection sites with a skin biopsy punch and processed individually for radiometric analysis. To this end the samples were digested for 18 h at 50° in 0·75 ml of NCS tissue solubilizer (Amersham, Searle Corp., Arlington, Heights, Ill.) and 0·25 ml of toluene. The material was neutralized with 20 μl of glacial acetic acid. Ten ml of a scintillator solution (Permablend® III, Packard Instrument Co., Downers Groove, Ill., 4·05 g/l toluene), were added followed by storage at +4° for 24 h. Radioactivity was determined in a liquid scintillation counter (Wallac 81,000). The ratio of radioactivity in the right and left ear samples of individual rats was expressed as an 'ear index'.

Ear swelling test

This was done immediately before ear biopsy in the same rats as the radiometric ear index determination. Ear thickness was measured like the flank skin tests before and after antigen injection. Increase in ear thickness (swelling) was calculated as a difference between the value measured before injection and at the given time intervals after it.

Measurement of serum antibody

Antibodies to BSA were measured by a precipitation technique (Weir, 1973). Sera were obtained by heart puncture at the time of killing of the rats for ear index tests. Half a millilitre of serum was added to an equal volume of 0·85% saline and serial two fold

dilutions were made in saline. This was followed by the addition of 10 μg of BSA in 0·5 ml of saline to each tube after which the tubes were incubated at +4° for 7 days. The titre was expressed as the reciprocal of the dilution in the last tube having a visible precipitate.

Lymphocytes

Thoracic duct lymphocytes (TDL) were obtained from donors during the first 24 h of lymph drainage. TDL collected from 6 donors were pooled, washed once in Hanks's balanced salt solution and resuspended in the same medium at $2{\cdot}5 \times 10^8$/ml.

Autoradiography

Ear biopsy specimens were fixed in 10% neutral formalin. They were dehydrated, embedded in paraffin and sectioned in thickness of 2–4 μm. Autoradiographs were prepared with AR 10 stripping film (Kodak, England), exposed 5 weeks at +4°, developed and stained with Giemsa solution.

Statistical analysis

Comparisons between paired groups were made by Student's *t*-test.

RESULTS

Specificity of the radiometric ear index test

Various *in vivo* assays used to measure delayed-type hypersensitivity and their *in vitro* correlates have been shown to be specific for the immunizing antigen (Turk, 1967; David & David, 1972). Therefore, it was important to establish the specificity of the ear index test at 24 h in classical delayed-type hypersensitivity, i.e. in animals immunized with soluble protein antigens in FCA. Accordingly, groups of 8 rats were immunized with 500 μg of BSA or 240 μg of DT in FCA and pulsed with [^{3}H]-thymidine on day 20. Twenty-four hours later they were injected either with 50 μg of BSA or 24 μg of DT in the pinna of the right ear and saline diluent in the pinna of the left ear. Ear biopsies were taken at 24 h and radioactivity in them determined. Table 1 indicates that substantial accumulation of radionucleotide at the antigen test site occurred only in rats immunized with the specific protein. Indexes near 1·0 were found in non-immunized rats, i.e. they did not react to the test proteins.

Table 1. Specificity of the radiometric ear index test at 24 h

Immunization *	Ear index†	
	BSA	DT
BSA	4·4±0·6‡	1·8±0·2
DT	1·2±0·2	4·1±0·6‡
Not immunized	0·9±0·1	1·2±0·1

* Rats were immunized either with 500 μg of bovine serum albumin (BSA) or 240 μg of diphtheria toxoid (DT) in Freund's complete adjuvant and tested on day 21 after immunization (see Method).

† Ratio of radioactivity in the right (test) and left (control) ears of rats 24 h after injection of 50 μg of BSA or 24 μg of DT in the pinna of the right ear and saline diluent in the pinna of the left ear. Means of 4± s.e.

‡ Significant ($P < 0{\cdot}001$) when compared with non-immunized group.

Sensitivity of the radiometric ear index test

A second experiment was designed to study the sensitivity of the 24 h ear index test by diluting out the antigen 10,000 fold beginning from 50 μg of BSA. The rats were similarly immunized with BSA and treated as in the previous experiment. As shown in Table 2, the test was found to be extremely sensitive. Positive indexes were induced with as little as 0·05 μg of BSA.

These experiments demonstrated the specificity and high sensitivity of the ear index test at 24 h after irritation with the specific antigen. The method is based on radioactive labelling of bone marrow precursors of monocytes. Monocyte derived macrophages subsequently accumulate at sites of inflammation, for instance in a DTH reaction (McCluskey, Benacerraf & McCluskey, 1963; Volkman & Gowans, 1965; Lubaroff & Waksman, 1967), whereas the cellular elements of 4 h skin reactions consist mainly of other cell types (Gell, 1961). Thus it was expected that at 4 h after antigen injection significant ear indexes would not be found. In the following experiment, the kinetics of the radiometric ear index reaction was studied.

Time course of the radiometric ear index reaction

Groups of rats were immunized with BSA and 21 days later tested with graded doses of BSA as before. Ear biopsies were obtained at 4, 24 and 48 h and

Table 2. Sensitivity of the radiometric ear index test at 24 h

Group*	Ear index †				
	50	5	0·5	0·05	0·005
Immunized	3·6±0·1‡	5·3±0·6‡	3·8±0·4‡	2·8±0·5‡	1·3±0·2
Control	1·0±0·1	1·0±0	1·2±0·1	0·9±0·1	1·2±0·3

* Rats were immunized with 500 μg of bovine serum albumin (BSA) in Freund's complete adjuvant and tested on day 21 after immunization (see Method). Control's were non-immunized rats.

† Ratio of radioactivity in the right (test) and left (control) ears of rats 24 h after injection of 50, 5, 0·5, or 0·005 μg of BSA in the pinna of the right ear and saline diluent in the pinna of the left ear. Means of 4–7±s.e.

‡ Significant ($P<0·01$) when compared with non-immunized group.

their radioactivity determined. As can be seen in Fig. 1., none of the antigen doses (50, 5, 0·5 or 0·05 μg of BSA) induced any positive reactions in normal rats. However, immunized rats tested with the three highest doses of antigen reacted with significant accumulations of radioactivity as early as at 4 h. As expected on the basis of previous experiments, all doses gave positive reactions at 24 h, and these were found to persist at high levels still at 48 h. The question now arises as to what is the mechanism of the unexpected positive reactions at 4 h. To solve this problem, the following experiments were done. First, a comparison was made with the results of other skin test assays.

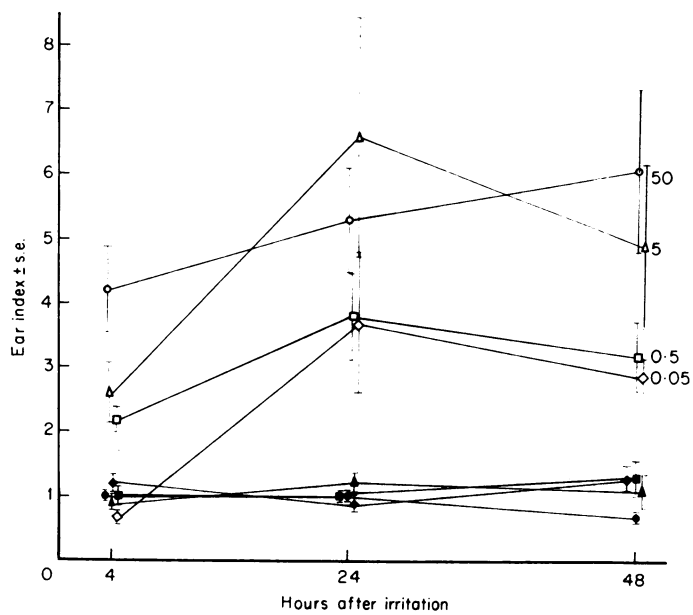

Figure 1. Curves describing the kinetics of the radiometric ear index reaction in BSA immunized rats. Animals which had been pulse labelled with [^{3}H]-thymidine were injected in the pinna of the ear with 50 (○), 5 (△), 0·5 (□) or 0·05 (◇) μg of BSA and ear biopsies taken at 4, 24 or 48 h (see text). The three highest doses of antigen induced positive radiometric ear indexes at all time points, whereas the rats failed to react to 0·05 μg of BSA at 4 h. Closed symbols denote the reactions elicited in normal rats. Means of 4–7±s.e.

Table 3. Sensitivity and time course of the flank skin test

Group*	BSA†	Skin reaction at 4 h		24 h		48 h	
		Diameter‡	Swelling§	Diameter‡	Swelling§	Diameter‡	Swelling§
Immunized	50	16±3	1·5±0·4¶	16±1	2·3±0·2¶	7±4	1·0±0·3¶
Control		0	0·1±0·1	3±0	0·1±0	0	0·3±0·1
Immunized	5	10±1	0·9±0·1¶	12±1	1·3±0·2¶	3±3	0·7±0·2
Control		4±1	0·2±0·1	0	0·3±0·2	0	0·2±0·1
Immunized	0·5	2±4	0·1±0	7±1	0·4±0·2	0	0·2±0·1
Control		2±1	0·2±0·1	2±1	0·1±0·1	0	0·2±0·1

* Rats were immunized with 500 μg of bovine serum albumin (BSA) in Freund's complete adjuvant and tested 21 days later. Control rats were not immunized.

† Micrograms of BSA in 0·1 ml of saline injected intradermally in the right flank.

‡ Diameter of erythema (mm). Positive reaction ≥ 10. Means of 5±s.e.

§ Increase in skin thickness (mm) from the value measured before injection. Means of 5±s.e.

¶ Swelling significant ($P<0·01$) when compared with non-immunized group.

Time course and sensitivity of the flank skin and ear swelling tests

Table 3 demonstrates the results of testing BSA immunized rats at 21 days after sensitization with the classical flank skin test by recording the diameter of erythema and the increase in skin thickness at the intradermal test site. A comparison to Table 2 shows that 100 times more antigen was needed to detect DTH with this assay than when using the radiometric ear index test, since 5 μg of BSA was the lowest antigen dose that gave either 4 or 24 h responses in the flank skin test.

Increase in skin thickness in response to an injection of the specific antigen can also be determined in such an easily accessible site as the pinna of the ear. This location, however, is not suitable for measurement of erythema because the margin of it in the pinna is often poorly defined. Table 4 shows the results of ear swelling measurements in the same rats as illustrated in Fig. 1 for radiometric ear index reactions. These two tests appeared to be similar in time course and sensitivity except at two important points. These were both 4 h reactions; one is the strong ear swelling induced by 50 μg of BSA and the other is the small but significant increase in ear thickness in response to 0·05 μg of BSA. Thus as a probe for Arthus reactivity as well as for DTH, ear thickness measurement appeared to be 100 times more sensitive than the flank skin test (Table 3).

It is well established that 4 h flank skin reactivity correlates with the presence of precipitating antibody to the immunizing antigen and can be transferred to normal recipients with immune serum (Gell, 1961; Kabat & Mayer, 1961) whereas DTH reactions are cell mediated (Turk, 1967). The following experiment was based on this knowledge and was designed to study in adoptively immunized rats the mechanism by which radiometric ear index and ear swelling reactions are born.

Comparison of radiometric ear index and ear swelling reactions in recipients of immune TDL or immune serum

The donors of TDL were immunized with BSA in FCA and their thoracic ducts cannulated 7 days after immunization. The donors of immune serum were selected among rats illustrated in Fig. 1. First, the sera were titrated using a precipitation technique. The mean anti-BSA antibody titres ± s.e. thus obtained for rats studied at 4, 24 and 48 h after ear testing were 14±1·2, 14±1·1 and 10±1·1, respectively. All nonimmunized rats had a titre of <4. Some of the immune sera having the highest antibody titres were pooled and used in serum transfer. This pooled serum had a titre of 16.

The recipients were radioactively labelled with a pulse of [^{3}H]-thymidine and 24 h later received either $4·4 \times 10^8$ TDL or 2 ml of pooled immune serum intravenously. Within 1 h of transfer they were ear

Table 4. Sensitivity and time course of the ear swelling test

Group*	BSA†	Ear swelling‡ at		
		4 h	24 h	48 h
Immunized	50	1·01±0·05§	0·78±0·06§	0·74±0·05§
Control		0·06±0·01	0·01±0	0
Immunized	5	0·37±0·08§	0·53±0·05§	0·52±0·08§
Control		0·07±0·01	0·04±0	0·03±0·01
Immunized	0·5	0·14±0·02§	0·33±0·03§	0·24±0·03§
Control		0·04±0·02	0·03±0·01	0·02±0·01
Immunized	0·05	0·12±0·02§	0·22±0·06§	0·23±0·06§
Control		0·05±0·01	0·02±0·01	0·01±0·01
Immunized	0·005	0·10±0·01	0·10±0·02	0·03±0·02
Control		0·06±0·01	0·07±0·02	0·01±0

* Rats were immunized with 500 μg of bovine serum albumin (BSA) in Freund's complete adjuvant and tested 21 days later. Control rats were not immunized.

† Micrograms of BSA in 0·02 ml of saline injected into the pinna of the right ear.

‡ Increase in the thickness (mm) of the right ear from the value measured before injection. Means of 4–7±s.e.

§ Significant ($P<0·01$) when compared with non-immunized group.

tested with 5 μg of BSA, a dose known to induce 4 h as well as 24 h reactivity in actively immunized rats (Table 4, Fig. 1). The results in Table 5 show that the recipients of TDL reacted positively at 24 h but not at 4 h in both ear index and ear swelling tests, a fact that shows the mediation of these 24 h responses by immune lymphocytes. In contrast, a positive response in ear swelling reactions was seen in the recipients of immune serum at 4 h only, whereas in ear biopsies successively performed for the same rats no significant accumulation of radioactivity was found at any time after irritation with BSA. It should be noted

Table 5. Comparison of radiometric ear index and ear swelling tests in recipients of TDL or serum from rats immunized with BSA

Treatment of recipients*	Ear index† at		Ear swelling‡ at	
	4 h	24 h	4 h	24 h
TDL§	1·0±0·1	2·2±0·2**	0·05±0	0·14±0·01**
Serum¶	1·4±0·2	1·6±0·2	0·48±0·03**	0·05±0·01
None	0·9±0·1	1·1±0·1	0·04±0	0·03±0

* Rats were given intravenously either $4·4\times10^8$ TDL or 2 ml of serum 1 h before ear testing, or were left untreated.

† Ratio of radioactivity in the right (test) and left (control) ears of recipients at given time intervals after injection of 5 μg of BSA into the pinna of the right ear and saline diluent into the pinna of the left ear. Means of 4±s.e.

‡ Increase in the thickness (mm) of the right ear from the value measured before injection. Means of 4±s.e.

§ Collected from donor rats 7 days after immunization with BSA in FCA.

¶ Pooled serum obtained 21 days after immunization of donor rats with BSA in FCA.

** Significant ($P<0·001$) when compared with values of untreated group.

Table 6. Flank skin reactivity and serum antibody in BSA immunized rats challenged with BSA

Group*	Challenge†	Skin reaction‡ at 4 h Diameter§	Skin reaction‡ at 4 h Swelling¶	Skin reaction‡ at 24 h Diameter§	Skin reaction‡ at 24 h Swelling¶	Serum antibody titre††
Immunized	BSA	13±3	0·9±0·1**	8±3	0·3±0·1	16±1·2
Immunized	Saline	14±1	1·0±0·1**	16±1	1·6±0·1**	9±1·1
Control	None	2±1	0·1±0·1	1±1	0·1±0·1	<4

* Rats were immunized with 500 μg of bovine serum albumin (BSA) and 240 μg of diphtheria toxoid (DT) in Freund's complete adjuvant. Control group was not immunized.

† Ten mg of BSA in 0·1 ml of saline, or saline alone, was injected intracutaneously 21 and 28 days after immunization. Rats were tested 7 days later.

‡ Skin reaction induced by 50 μg of BSA at given time intervals after intracutaneous injection.

¶ Diameter of erythema (mm). Positive reaction ⩾10 mm. Means of 4±s.e.

§ Increase in skin thickness (mm) from the value measured before injection. Means of 4±s.e.

** Swelling significant ($P<0·001$) when compared with the control group.

†† Anti-BSA antibody titre measured by a precipitation technique (Mean ±s.e. of 16).

that the precipitating anti-BSA antibody titres of recipients of immune serum and immune TDL were 8 and <4, respectively.

These results demonstrated an operational difference at 4 h in ear swelling and radiometric ear index tests. The former measured a reaction that fulfilled the criteria for Arthus reactivity, i.e. it could be transferred to normal recipients with serum containing precipitating antibody, whereas the latter did not have an antibody mediated component. In the following experiment the influence of desensitization of DTH on these reactions was studied.

Desensitization of DTH

Doses of specific antigen given to previously sensitized rats or guinea-pigs lead to a state of unreactivity called desensitized DTH (Uhr & Pappenheimer, 1958; Kostiala & Kosunen, 1972; Poulter & Turk, 1976). Table 6 shows a typical example of this phenomenon in rats primarily sensitized with BSA and DT in FCA. At 21 and 28 days after immunization some of the rats were given intracutaneous boosters of 10 mg of BSA in saline whereas others received saline alone. All of the rats were skin tested intradermally in the flank with 50 μg of BSA 7 days later still. Positive Arthus and DTH reactions were found in recipients of saline whereas those reinjected with BSA had only 4 h reactivity. The persistence of antibody mediated reactivity was also seen in the titres of serum anti-BSA antibody which were at least as high in the group challenged with BSA as in that treated with saline alone.

A similar panel of rats was tested in the pinna of the ear instead of flank skin. To study the specificity of DTH desensitization at this test site, half of the rats were injected in the pinna of the right ear with BSA while the rest were similarly tested with DT, the second antigen they had been immunized with. Due to the higher sensitivity of the pinna of the ear as a test site, lower antigen concentrations than in the flank were used (0·5 μg of BSA and 0·24 μg of DT). As shown in Table 7 ear swelling reactions to BSA gave the same results as flank skin tests, i.e. a large dose of BSA injected in saline led to desensitization of DTH whereas it did not significantly change 4 h reactivity to BSA. In addition, the specificity of this phenomenon was shown in rats tested with the unrelated antigen DT, inasmuch as these 24 h reactions were positive.

The same rats used for ear swelling measurements were also processed for the radiometric ear index assay. The results in Table 7 show that 24 h ear index reactions followed exactly the pattern of desensitized DTH seen above. Another interesting finding of this experiment was the low or insignificant accumulation of radioactivity in the test site at 4 h in any of the groups. Ear swelling reactions, however, were positive and the serum precipitating

Table 7. Radiometric ear index and ear swelling reactions in BSA immunized rats challenged with BSA

Group*	Challenge†	Ear index‡				Ear swelling§			
		BSA		DT		BSA		DT	
		4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
Immunized	BSA	1·6±0·2	1·8±0·2	1·8±0·3	2·6±0·4¶	0·14±0·02¶	0·08±0·03	0·14±0·01¶	0·36±0·03¶
Immunized	Saline	1·5±0·2	4·2±0·9¶	1·5±0·1	3·9±0·7¶	0·16±0·01¶	0·36±0·03¶	0·15±0·01¶	0·21±0·02¶
Control	None	1·1±0·2	1·3±0·2	1·2±0·1	1·2±0·1	0·04±0·02	0·03±0·01	0·02±0·01	0·03±0·01

* Rats were immunized with 500 μg of bovine serum albumin (BSA) and 240 μg of diphtheria toxoid (DT) in Freund's complete adjuvant. Control group was not immunized.

† Ten mg of BSA in 0·1 ml of saline, or saline alone, was injected intracutaneously 21 and 28 days after immunization. Rats were tested 7 days later.

‡ Ratio of radioactivity in the right (test) and left (control) ears of rats 4 and 24 h after injection of 0·5 μg of BSA or 0·24 μg of DT in the pinna of the right ear and saline diluent in the pinna of the left ear. Means of 4–6± s.e.

§ Increase in the thickness (mm) of the right ear from the value measured before injection. Means of 4–6± s.e.

¶ Significant ($P < 0·01$) when compared with the control group.

anti- BSA antibody titres similar to those seen in Table 6. This finding adds further evidence to the dissociation of reactivity measured in the radiometric ear index assay and antibody mediated mechanisms. In addition, a comparison with Fig. 1 shows that the marginal response to low antigen doses at 4 h in the radiometric ear index test seems to vary unpredictably from experiment to experiment.

Autoradiography

The localization of radioactivity at 4 or 24 h in the test site in the pinna of the ear can be directly studied by performing autoradiographs. Rats immunized with BSA and tested on day 21 with 5 μg of BSA as before showed a cellular infiltrate at the test sites already as early as 4 h after irritation with antigen. It consisted mostly of unlabelled granulocytes with a minority of mononuclear cells some of which were labelled. A totally different picture was seen at 24 h, the abundant cellular infiltrate consisting mainly of monocytes and macrophages about 50% of which were labelled.

DISCUSSION

According to the present results, the pinna of the ear should be preferred to the usual flank skin as a DTH test site in the rat. Assays performed in the pinna of the ear, whether radiometric ear index determination or increase in ear thickness, were 100 times more sensitive than flank skin tests and detected DTH with as little as 0·05 μg of BSA in appropriately sensitized animals (Tables 2, 3, 4, Fig. 1). This is in agreement with the results of Robinson & Naysmith (1976) who, when working with mice, found ear swelling determinations more sensitive than footpad reactions. Vadas *et al.* (1975) and Miller, Vadas, Whitelaw & Gamble (1975) have used a similar radioisotopic ear method in the mouse and found it to demonstrate the existence of a state of delayed-type hypersensitivity.

Ear index determination which is based on the recovery of macrophage bound radioactivity from the test site in the pinna of the ear, is a more tedious and expensive assay system than the measurement of increase in ear thickness using special calipers. On the other hand, practice is needed to obtain reproducible results with the latter which also should be performed by one person throughout the study, whereas the former is more objective. However, the most critical points in favour of the radiometric ear index determination were derived from experiments in which the mechanisms of these two tests were studied.

The rats used in the present investigation were immunized with BSA in FCA and as a result had precipitating anti-BSA antibody in their sera. When skin tested in the flank they showed Arthus reactivity which appeared as a positive skin reaction 4 h after irritation with the specific antigen (Table 3). This reaction type is known to be mediated by antigen–antibody complexes precipitating at the local test site followed by mainly granulocytic infiltration and oedema (Kabat & Mayer, 1961). There are sound reasons for thinking that the ear swelling reactions at 4 h were also caused by this serum antibody mediated mechanism. In fact, this was shown to be true in an experiment in which positive ear swelling reactions were induced in normal recipients after transfer of a precipitating anti-BSA serum obtained from donors that themselves showed Arthus reactivity (Table 5).

The finding of significantly high radiometric ear indexes as early as 4 h after irritation with a high dose of antigen (Fig. 1) was unexpected, inasmuch as the test is based on labelling of precursors of macrophages which are an inconspicous element of 4 h reactions (Gell, 1961). However, several possibilities should be considered in order to explain this.

It is highly unlikely that the positive indexes at 4 h were derived from radioactivity remaining in tissue fluids since [^{3}H]-thymidine remains available in the organism for only a short period after injection (Volkman & Gowans, 1965). Numerous granulocytes accumulate at 4 h in the skin test site due to Arthus reactivity but are not labelled due to timing of the isotope injection (Volkman & Gowans 1965). On the other hand, replicating lymphocytes of the type which is known to enter centres of inflammation are labelled by a radioactive pulse (Koster & McGregor, 1971) and can therefore contribute to the radioactivity recovered. These cells, however, are only a minor part of the cellular infiltrate in 4 h reactions (Gell, 1961; Kosunen, Waksman, Flax & Tihen, 1963). Another possibility that remains is that the radioactivity carried by the few macrophages arriving early at the test site (Kosunen *et al.*, 1963; McCluskey *et al.*, 1963) contributed to the positive radiometric indexes in this sensitive assay.

Several lines of evidence point to a cell mediated

mechanism. Firstly, on the basis of earlier experiments it is known that labelled macrophages arrive early at sites of inflammation in skin window preparations in similarly labelled rats (Volkman & Gowans, 1965). In fact, in the present study autoradiographs revealed labelled mononuclear cells to be present locally at the test sites as early as 4 h after irritation with the specific antigen. An important result is that 4 h radiometric ear indexes were negative in recipients of precipitating immune serum although ear swelling reactions at 4 h were positive (Table 5). This finding strongly speaks against antibody as the mediator of 4 h radiometric ear reactions. Still further evidence for the idea comes from experiments in which rats sensitized so as to show DTH to BSA were desensitized with repeated BSA injections. Tables 6 and 7 show that radiometric ear index values in desensitized rats did not correlate with serum antibody titres or 4 h reactions as measured in the flank skin or ear swelling tests.

The specific desensitization of DTH revealed by the radiometric ear index test is in agreement with the finding of Poulter & Turk (1976) who described the inability of macrophages of desensitized animals to respond to lymphokine and thus accumulate and stay at the test site.

Although typically an early lesion, antibody mediated Arthus reaction when intense, may be maximal at any time from 2 to 24 h (Gell, 1961). Thus traces of this reactivity can badly interfere with the results of DTH tests as far as timing is concerned. As an assay system the radiometric ear index test thus represents a measure of 'pure' DTH since these indexes do not arise by a serum antibody mediated mechanism, provided that testing is done with a low enough dose of antigen. If unnecessarily large amounts of antigen are used to test animals having high precipitating antibody titres, the strong early inflammation leads to widespread damage and leakage of cellular elements including labelled macrophages which subsequently may be picked up as positive 4 h reactions in this extremely sensitive assay.

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REFERENCES

AXELRAD M.A. (1968) Supression of delayed hypersensitivity by antigen and antibody. Is a common precursor cell responsible for both delayed hypersensitivity and antibody formation? *Immunology*, **15**, 159.

CRUM E.D. & MCGREGOR D.D. (1976) Induction of delayed-type hypersensitivity with BCG-associated proteins. *Immunology*, **30**, 497.

DAVID J.R. & DAVID R.R. (1972) Cellular hypersensitivity and immunity. Inhibition of macrophage migration and the lymphocyte mediators. *Progr. Allergy*, **16**, 300.

FLAX M.H. & WAKSMAN B.H. (1962) Delayed cutaneous reactions in the rat. *J. Immunol.* **89**, 496.

FRANCO M.F. & MORLEY J. (1976) Use of ^{125}I-labelled albumin for the detection and measurement of delayed-hypersensitivity reactions in the mouse. *J. Immunol. Methods*, **11**, 7.

GELL P.G.H. (1961) Cytologic events in hypersensitivity reactions. In: *Cellular and humoral aspects of the hypersensitivity states* (ed. by H.S. Lawrence), p. 43. Paul B. Hoeber, New York.

KABAT E.A. & MAYER M.M. (1961) *Experimental Immunochemistry*, 2nd edn. Charles C. Thomas, Springfield, Illinois.

KOSTER F.T. & MCGREGOR D.D. (1971) The mediator of cellular immunity. III. Lymphocyte traffic from the blood into the inflammed peritoneal cavity. *J. exp. Med.* **133**, 864.

KOSTIALA A.A.I. & KOSUNEN T.U. (1972) Delayed hypersensitivity, macrophage migration and antibodies in guinea-pigs immunized with diphtheria toxoid. II. Macrophage cytophilic antibody to diphtheria toxoid in desensitized animals. *Scand. J. Immunol.* **1**, 153.

KOSTIALA A.A.I. & MCGREGOR D.D. (1975) The mediator of cellular immunity. IX. The relationship between cellular hypersensitivity and acquired cellular resistance in rats infected with *Listeria monocytogenes*. *J. exp. Med.* **141**, 1249.

KOSTIALA A.A.I., MCGREGOR D.D. & LOGIE P.S. (1975) Tularaemia in the rat. I. The cellular basis of host resistance to infection. *Immunology*, **28**, 855.

KOSUNEN T.U., WAKSMAN B.H., FLAX M.H. & TIHEN W.S. (1963) Radioautographic study of cellular mechanisms in delayed hypersensitivity. I. Delayed reactions to tuberculin and purified proteins in the rat and guinea-pig. *Immunology*, **6**, 276.

LEFFORD M.J. (1974) The measurement of tuberculin hypersensitivity in rats. *Int. Arch. Allerg. Appl. Immunol.* **47**, 570.

LUBAROFF D.M. & WAKSMAN B.H. (1967) Delayed hypersensitivity: Bone marrow as the source of cells in delayed skin reactions. *Science*, **157**, 322.

MCCLUSKEY R.T., BENACERRAF B. & MCCLUSKEY J.W. (1963) Studies on the specificity of the cellular infiltrate in delayed hypersensitivity reactions. *J. Immunol.* **90**, 466.

MILLER J.F.A.P., VADAS M.A., WHITELAW A. & GAMBLE J. (1975) A radioisotopic method to measure delayed type hypersensitivity in the mouse. II. Cell transfer studies. *Int. Arch. Allerg. Appl. Immunol.* **49**, 693.

POULTER L.W. & TURK J.L. (1976) Changes in macrophages *in vivo* induced by desensitization. *Cellular Immunology*, **23**, 171.

SABOLOVIC D., BEUGNOT M-C., DUMONT F. & BUJADOUX M. (1972) A new method to measure the specific cellular component of a delayed hypersensitivity response in the ear of the mouse. *Eur. J. Immunol.* **2**, 604.

TURK J.L. (1967) *Delayed hypersensitivity*. North Holland Publishing Company, Amsterdam.

UHR J.W. & PAPPENHEIMER A.M. (1958) Delayed hypersensitivity. III. Specific desensitization of guinea-pigs sensitized to protein antigens. *J. exp. Med.* **108**, 891.

VADAS M.A., MILLER J.F.A.P., GAMBLE J. & WHITELAW A. (1975) A radioisotope method to measure delayed type hypersensitivity in the mouse. I. Studies in sensitized and normal mice. *Int. Arch. Allerg. Appl. Immunol.*, **49**, 670.

WEIR D.M. (1973) *Handbook of experimental immunology. Volume* 1. *Immunochemistry*, 2nd. edn. Blackwell Scientific Publications, Oxford.

VOLKMAN A. & GOWANS J.L. (1965) The production of macrophages in the rat. *Brit. J. exp. Path.* **46**, 50.