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The Induction of Delayed Hypersensitivity by Macrophage-associated Antigen

THE ROLE OF MACROPHAGE CYTOPHILIC ANTIBODY

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Summary. The regulation of the immune responses by antibody administered passively on peritoneal exudate cells (PEC) was studied in guinea-pigs. The immunogenicity of sheep red blood cells (SRBC) associated with PEC for the induction of delayed hypersensitivity (DH) to soluble erythrocyte antigen was enhanced when PEC were incubated with anti-SRBC antibody. In contrast, the antibody response to SRBC was depressed. This phenomenon was only observed with specific antibody and was partially blocked when PEC covered with anti-SRBC antibody were incubated with rabbit anti-guinea-pig globulin serum. Comparable amounts of anti-SRBC antibody injected separately had no enhancing effect. Anti-SRBC sera from which the cytophilic antibody activity had been removed were inactive. PEC-associated SRBC were usually more immunogenic than the same amount of 'free' SRBC for the induction of delayed hypersensitivity and always more immunogenic for antibody production.

These observations suggested that macrophage cytophilic antibody might be involved in the regulation of the immune response and play a role in the preferential induction of delayed hypersensitivity in the studied system.

INTRODUCTION

There is evidence that the macrophage is an important cell in the inductive phase of the humoral response both *in vitro* (Hoffman, 1970; Shortman and Palmer, 1971) and *in vivo* (Unanue and Askonas, 1968). Macrophage-associated antigen is especially effective in priming mice and guinea-pigs for antibody synthesis (Mitchison, 1969; Seeger and Oppenheim, 1972). Macrophage-bound antigen is also more immunogenic than 'free' antigen for the induction of delayed type hypersensitivity. This is true for both particulate and soluble antigens (Bloch and Nordin, 1960; Pearson and Raffel, 1971; Asherson, Allison and Zembala, 1971). Macrophages are also required in *in vitro* cellular responses, e.g. sensitization to transplantation antigen (Wagner, Fekhnann, Boyle and Schrader, 1972) and potentiation of the blast response of sensitized lymphocytes (Seeger and Oppenheim, 1970). Macrophage-associated antigen appears to interact predominantly

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with thymus-dependent (T) lymphocytes prior to involvement of other immunocompetent cells (Seeger and Oppenheim, 1972; Kunin, Shearer, Globerson and Feldman, 1972).

Little is known about the factors which influence the immunogenicity of macrophage-associated antigen. It has been suggested that antigen which is highly degraded during catabolism within the macrophage favours delayed hypersensitivity response (Pearson and Raffel, 1971) and that small amounts of membrane-bound antigens are highly immunogenic for the induction of the humoral antibody response (Cruchaud and Unanue, 1971). Passively administered antibody suppresses the antibody formation specifically (Uhr and Möller, 1968). Liew and Parish (1972) have recently found that antibody-mediated suppression of humoral response leads to the concomitant enhancement of delayed hypersensitivity skin reactions in rats.

This paper describes the role of macrophage cytophilic antibody in enhancing delayed hypersensitivity responses and depressing antibody response. It shows that SRBC associated with PEC are more immunogenic than 'free' SRBC. Treatment of the macrophages with specific cytophilic antibody has two effects: on the one hand it increases the delayed hypersensitivity response to soluble erythrocyte antigen, while on the other hand it depresses antibody production.

MATERIALS AND METHODS

Animals

Colony-bred albino guinea-pigs of both sexes weighing 300–500 grams were used.

Antigens

SRBC stored in Alsever's solution in the cold were washed four times before use. To increase phagocytosis by macrophages, SRBC were damaged by heating at 50° for 30 minutes. Urea extracts of SRBC or horse erythrocytes (HRBC), prepared according to the method of Boyden (1964), were used for skin testing. For *in vitro* experiments ultrasonicated soluble erythrocyte antigen (sSRBC) was employed (Waterson, 1970). The activity of soluble antigens was assessed in haemagglutination inhibition tests against four HA units of anti-erythrocyte antibody. Diphtheria toxoid (DT) was obtained from the Laboratory of Sera and Vaccines, Cracow; 2000 Lf/mg protein.

^{51}Cr labelling of erythrocytes

100 μCi of sodium [^{51}Cr]chromate (Institute of Nuclear Studies, Warsaw; specific activity 40–100 μCi/μg) was added to 1 ml of packed SRBC, resuspended to make a 10 per cent solution in phosphate-buffered saline (PBS), incubated for 1 hour at 37°, and then washed several times.

Iodination

γ_2-anti-SRBC globulin or sSRBC antigen were labelled with ^{131}I by chloramine T method at about 1 μCi/μg (McConahey and Dixon, 1966). Iodide was removed by dialysis.

Preparation of antisera

Guinea-pigs were immunized with 0·2 ml of either 30 per cent solution of SRBC or 100 Lf of DT mixed with an equal volume of Freund's complete adjuvant (Freund's incomplete adjuvant, Difco, with added human tubercle bacilli, 1 mg/ml final concen-

tration) and 0·1 ml was injected into each of the four footpads. Three weeks later animals were bled by cardiac puncture under ether anaesthesia. Antisera with cytophilic antibody (Cab) titre at least 1:320 were used, except in experiment 2 (Table 2). Anti-SRBC γ_2-globulin was isolated by DEAE–cellulose chromatography with 0·01 M pH 8·0 phosphate buffer. Rabbit anti-guinea-pig globulin sera (AGPGS) were produced by immunization with guinea-pig globulin obtained by three precipitations with sodium sulphate in FCA followed by intravenous injections of alum-precipitated antigen. For details see Zembala and Asherson (1970).

Antibody determination

Haemagglutinating (HA), haemolytic and cytophilic antibody was determined following Davey, Asherson and Stone (1971) with minor modifications: (a) HA was read within 3 hours at room temperature; (b) Cab test was performed in Leighton tubes. Immune sera were incubated with macrophages at 4°; 0·2 per cent SRBC were added to antibody-covered macrophages; tubes were read under microscope after further incubation for 1 hour at room temperature. For anti-DT antibody determination DT coupled to SRBC by BDB method (Weir, 1967) was used.

Peritoneal exudate cells (PEC)

Peritoneal exudates were induced by injection of 20 ml of liquid light paraffin into the peritoneal cavity of normal guinea-pigs 5 days beforehand. In one experiment (Table 3) PEC from animals sensitized to SRBC were also used. The cells were harvested by washing out the peritoneal cavity with balanced salt solution (BSS) containing 10 iu of heparin. The cells were recovered by centrifugation and washed.

Incubation of cells

PEC were incubated either with antisera or normal guinea-pig serum (NGPS) diluted 1:2 in BSS at the ratio $2{\cdot}5 \times 10^7$ cells/ml at 4° for 1 hour, washed, incubated with ^{51}Cr-labelled SRBC for 90 minutes with shaking at 37°. In the other experiments PEC were given SRBC prior to the addition of antisera. The extracellular SRBC were removed by the incubation of the cell mixture with Tris-buffered ammonium chloride buffer (Boyle, 1968) for 10 minutes at 37°, centrifuged and washed. The amount of ingested SRBC was ascertained by the determination of radioactivity of the cell sample in a well type gamma counter. In some experiments Cab-coated PEC were incubated with AGPGS and anti-γ_2-globulin sera for 30 minutes at room temperature and then washed. Cells incubated in normal rabbit serum (NRS) served as a control. The cells were 85–90 per cent viable as judged by Trypan Blue (final concentration 0·05 per cent) dye exclusion test.

Cell injections

Comparable amounts of either PEC-associated SRBC treated with different antisera or NGPS or free antigen were injected intraperitoneally into normal recipients in 5 ml balanced salt solution (BSS). Guinea-pigs were skin tested at 7 days and retested 14 and sometimes 21 days later. The sera from these animals were collected for antibody determination on day 16.

Skin test

Guinea-pigs were injected with 0·1 ml of urea extract of SRBC or HRBC, diluted 1:2

in saline. The induration, i.e. the increase in thickness of a double-fold of skin, was measured with a dial gauge (Schnelltaster) in units of 10^{-2} cm at 4, 24 and 48 hours. The erythema was measured in mm at 24 hours with simultaneous observation of the intensity of the reaction expressed in four degree scale; from (+)= light pink to (++++)= haemorrhage. There were five to eight animals per group except in the experiment recorded in Table 5 (four to five animals per group).

Absorption of the immune serum with PEC

Anti-SRBC serum was incubated with normal PEC at the ratio 5×10^7 cells/ml at 4° for 1 hour. This procedure was repeated seven times until undiluted serum showed no rosette formations. HA titre in absorbed serum was 1:2560.

Pepsin treatment

Pepsin treatment of anti-SRBC globulins obtained by precipitation with sodium sulphate was performed according to Nisonoff, Wissler, Lipman and Woernley (1960). $F(ab')_2$ fragments were purified on a Sephadex G-200 column.

Iodoacetamide (IAA) treatment

PEC were treated with 0·05 M iodoacetamide (Koch Light Labs, Colnbrook, Bucks.) for 1 hour at 37° and washed twice in BSS (Howard and Benacerraf, 1966). The cells were subsequently incubated with anti-SRBC serum.

In vitro *observations*

Binding of ^{131}I-labelled Cab by PEC. PEC (5×10^6) were incubated in 2 ml BSS containing variable amounts of ^{131}I-labelled γ_2-anti-SRBC antibody for ½, 1, 2 and 3 hours. The cells were then centrifuged, washed ten times and cell-associated radioactivity was determined.

Uptake of ^{131}I-labelled antigen by Cab-coated PEC. PEC were incubated for 1 hour at 4° with 500 μg of γ_2-anti-SRBC antibody/10^6 cells. After washing the cells were resuspended in TC 199 medium supplemented with 1 per cent NGPS and incubated at 37° on a roller (20 rpm). At different intervals PEC were washed and 50 μg of ^{131}I-labelled soluble ultrasonicated erythrocyte antigen was added to each tube. The reaction was carried out overnight at 4°. The cells were then washed ten times and the radioactivity was recorded. In parallel, macrophages which had previously been treated with anti-SRBC antibody were kept in Leighton tubes with flying coverslips at 37°. These cells were tested at different intervals for their ability to form SRBC rosettes.

RESULTS

THE EFFECT OF ANTISERUM ON THE IMMUNOGENICITY OF SRBC ASSOCIATED WITH MACROPHAGES AND THE EFFECT OF ANTI-GUINEA PIG GLOBULIN SERA

Normal, oil-induced PEC were allowed to phagocytose ^{51}Cr-labelled SRBC. The unphagocytosed antigen was removed by lysis, the cells were washed and incubated with either guinea-pig anti-SRBC serum, or anti-DT serum or normal serum (NGPS). Comparable amounts of PEC-SRBC or 'free' erythrocytes were injected into the peritoneal cavity of normal guinea-pigs which were skin-tested 7 days later with urea extract of

SRBC (or HRBC). The induration and diameter of skin reactions at 4, 24 and 48 hours were recorded.

Experiment 1 (Table 1) shows that erythrocytes associated with PEC and subsequently treated with anti-SRBC antibody exhibited a significantly greater ability to induce delayed skin reactions than SRBC-PEC incubated with an unrelated antiserum (anti-DT) or NGPS. PEC-associated antigen (see also experiments 2 and 3, Table 1) was usually,

TABLE 1

SKIN REACTIONS IN GUINEA-PIGS IMMUNIZED WITH PE-ASSOCIATED SRBC OR FREE SRBC

Experiment	Antigen	Mean induration at 4 hours	Mean induration at 24 hours	Mean induration at 48 hours	Mean diameter at 24 hours
1	PEC-SRBC+NGPS	3·6±0·8	3·1±1·4	5·8±0·4	11·2++
	PEC-SRBC+anti-SRBC	3·6±0·8	8·3±3·8	9·0±2·3	17·1+++
	PEC-SRBC+anti-DT	4·2±0·1	3·1±0·7	3·3±0·7	−ve
	PEC-SRBC+anti-SRBC +AGPGS	2·4±0·5	3·6±1·2	3·2±0·9	10·0+
	PEC-SRBC+anti-SRBC +NRS	2·8±0·6	6·6±1·9	5·7±1·7	14·0++
	SRBC 1×10^8	2·2±0·8	1·9±1·1	1·7±0·2	−ve
	Nil	1·4±0·7	0·8±0·8	0·4±0·2	−ve
2	PEC+anti-SRBC+NRS +SRBC	2·4±0·5	10.0±1·8	10·0±1·7	19·2+++
	PEC+anti-SRBC+ AGPGS+SRBC	2·0±0·7	2·8±2·1	3·0±1·2	9·1++
	PEC+anti-DT+SRBC	2·2±0·9	4·5±1·4	3·7±0·8	14·0+
	SRBC $1·7\times10^8$	1·7±0·4	2·5±1·5	2·2±0·9	−ve
	Nil	2·1±1·4	0·4±0·2	0·8±0·6	−ve
3	PEC+anti-SRBC (21 day)+SRBC	2·7±0·5	8·6±3·9	7·5±3·6	15·4+++
	PEC+anti-SRBC (10 day)+SRBC	2·5±0·7	4·8±0·9	5·3±1·2	11·2++
	PEC+anti-DT+SRBC	2·7±0·4	2·5±0·4	1·1±0·6	11·0±
	PEC+NGPS+SRBC	2·5±0·1	1·7±0·4	2·7±0·7	−ve
	SRBC 1×10^8	3·5±0·5	1·9±1·4	2·9±0·1	−ve
	Nil	1·1±0·3	0·6±0·1	0·5±0·1	−ve

Guinea-pigs were injected with normal PEC which had phagocytosed SRBC and were then coated with different antisera (experiments 1 and 3). In experiment 2, PEC were incubated with specific (anti-SRBC) or unrelated (anti-DT) antiserum and then allowed to phagocytose SRBC. The number of 'free' SRBC shown is the same as the amount of SRBC associated with PEC. The figures show the mean diameter at 24 hours and the mean induration at 4, 24 and 48 hour skin reaction following challenge 7 days after immunization with urea extract of SRBC ± standard deviation.

but not always, more immunogenic than 'free' antigen. Table 1 also shows that the enhancing effect of the specific immune serum was diminished when the PEC were subsequently treated with AGPGS. The enhancement of the immunogenicity of the antigen was also observed when PEC were coated with specific antibody prior to the addition of SRBC. The blocking effect of AGPGS was also seen (experiment 2, Table 1). Experiment 3 (Table 1) demonstrates that immune serum obtained 21 days after immunization of guinea pigs with SRBC in FCA (Cab titre 1:640, HA titre 1:5180) was more efficient in producing the enhancement of the immune response than serum from animals 10 days after immunization (Cab titre 1:20, HA 1:1280).

When guinea-pigs injected with SRBC-PEC were retested with soluble erythrocyte antigen 14 days later, the differences between the groups were less. The 4-hour skin

reactions were much higher and the control background was also higher (Table 2). These differences were also apparent when guinea-pigs were retested 3 weeks, instead of 2 weeks, after antigen administration.

Guinea-pigs immunized with SRBC did not show skin reactions when tested with urea extract of HRBC.

TABLE 2

SKIN REACTIONS IN GUINEA-PIGS IMMUNIZED WITH PEC-ASSOCIATED ANTIGEN RETESTED WITH SOLUBLE ERYTHROCYTE ANTIGEN

Experiment	Antigen	Mean induration at 4 hours	Mean induration at 24 hours	Mean induration at 48 hours	Mean diameter at 24 hour
1	PEC+anti-SRBC+NRS +SRBC	6·6±1·2	13·3±7·4	10·1±8·3	14·2+++
	PEC+anti-SRBC+ AGPGS+SRBC	7·8+2·9	8·3+1·6	4·3±0·3	9·1++
	PEC-SRBC+anti-SRBC	5·8±2·5	10·8±6·6	8·9±6·1	16·0+++
	PEC+anti-DT+SRBC	8·7±1·8	5·9±4·2	4·8±3·2	7·1+
	SRBC 1×10^8	8·0±2·5	5·3±3·6	5·4±2·2	8·0±
	Nil	5·5±2·4	4·6±0·4	4·8±1·9	5·1±
2	PEC-SRBC+anti-SRBC	10·0±3·1	11·7±5·1	8·4±3·2	21·2+++
	PEC-SRBC+NGPS	5·4±2·4	5·6±2·2	3·8+1·7	8·0++
	SRBC 1×10^8	7·5±2·9	4·0±0·6	2·9±0·5	−ve
	Nil	3·4±2·0	2·9±0·8	1·4±0·9	−ve

Guinea-pigs injected with 'free' and PEC-associated SRBC. PEC were treated with different antisera. Animals were tested 14 days after immunization.

It was concluded that SRBC associated with PEC which had been covered with specific antibody (presumably cytophilic) showed a significantly increased ability to induce delayed hypersensitivity reactions. This could be partially blocked by treatment with rabbit AGPG serum. The phenomenon was immunologically specific as antisera with high titre of Cab to an unrelated (DT) antigen were inactive.

THE IMMUNOGENICITY OF SRBC ASSOCIATED WITH PEC TAKEN FROM SENSITIZED DONORS

In the next experiment PEC from guinea-pigs immunized with SRBC in FCA 21 days beforehand were incubated with ^{51}Cr-labelled SRBC. Some cells were subsequently incubated with anti-SRBC serum or treated with AGPG serum. Table 3 shows that antigen ingested by PEC from sensitized donors (known to carry Cab on their surface, as judged from rosette formation ability) was more immunogenic than the same amount of SRBC in PEC obtained from normal donors. It is of interest that the addition of anti-SRBC serum to these cells did not enhance and sometimes slightly decreased the immunogenicity of SRBC associated with PEC from sensitized animals. Treatment of PEC with AGPG serum partially inhibited the enhancing effect.

THE EFFECT OF REMOVING CYTOPHILIC ANTIBODY FROM THE IMMUNE SERUM AND THE ACTIVITY OF F(ab')$_2$ FRAGMENTS

SRBC-PEC were incubated in anti-SRBC sera which had been adsorbed with PEC to remove Cab or treated with pepsin to produce F(ab')$_2$ fragments (which are not

TABLE 3

TWENTY-FOUR-HOUR SKIN REACTIONS IN GUINEA-PIGS IMMUNIZED WITH SRBC ASSOCIATED WITH PEC TAKEN FROM GUINEA-PIGS SENSITIZED TO SRBC

Antigen	Mean induration	Mean diameter
PEC-SRBC	4.8±1·3	17·4+++
PEC-SRBC+Anti-SRBC	2·9±2·2	8·2++
PEC-SRBC+anti-SRBC+AGPGS	1·3±0·2	7·4+
PEC-SRBC+AGPGS	1·6±0·1	13·1++
PEC+anti-SRBC+SRBC	4·2±0·2	15·0++
Normal PEC-SRBC	1·6±0·8	8·8+
Nil	0·5±0·2	—ve

PEC obtained from guinea-pigs immunized with SRBC in FCA (21 days beforehand) were incubated with SRBC and then with different antisera. SRBC associated with PEC from normal animals were regarded as a control. The number of SRBC injected were 7×10^7. The figures show 24-hour skin reactions on a challenge 7 days later.

cytophilic). Some PEC were treated with iodoacetamide prior to the addition of immune serum.

Table 4 shows that SRBC-PEC which were coated with anti-SRBC antibody exhibited enhanced ability to induce skin reactions as compared with SRBC-PEC treated with NGP serum. In contrast, immune sera deprived of Cab activity either by absorption or by removal of Fc fragments lost this capacity and PEC treated with these sera gave rise to the reactions comparable to those elicited by SRBC-PEC incubated in normal serum. Iodoacetamide which affects the macrophage receptor for Cab also abolished the effect of immune serum. Similar results were obtained in two additional unreported experiments. These differences were also observed when animals were retested 14 days after antigen injection.

It was concluded that the enhancing effect of anti-SRBC serum was only observed when Cab was present in the immune serum.

TABLE 4

TWENTY-FOUR-HOUR SKIN REACTIONS IN GUINEA-PIGS IMMUNIZED WITH PEC ASSOCIATED SRBC. THE EFFECT OF ANTI-SRBC SERA DEPRIVED OF CYTOPHILIC ACTIVITY

Antigen	Challenge on day			
	7		14	
	Mean induration	Mean diameter	Mean induration	Mean diameter
PEC-SRBC+NGPS	3·8±1·7	7·4+	3·0±1·7	−ve
PEC-SRBC+anti-SRBC	9·7±1·8	19·1+++	11·7±3·2	21·0+++
PEC-SRBC+anti-SRBC (absorbed)	4·9±1·7	5·7+	4·4±1·9	14·0++
PEC-SRBC+anti-SRBC (pepsin-treated)	4·4±0·9	8·2+	3·1±1·0	11·5+
PEC-SRBC+IAA+ anti-SRBC	4·3±0·7	7·0+	4·6±1·2	±
SRBC 1×10^8	2·7±1·3	−ve	2·9±1·2	±
Nil	1·0±0·8	−ve	1·4±0·6	−ve

Guinea-pigs were immunized with SRBC-PEC which had been incubated with anti-SRBC serum either untreated or absorbed with PEC to remove Cab or treated with pepsin. Some PEC were treated with iodoacetamide (IAA) prior to anti-SRBC serum addition. Figures show 24-hour skin reactions at 7 and 14 days after immunization.

THE EFFECT OF ANTI-SRBC ANTIBODY INJECTED ON PEC OR SEPARATELY

Guinea-pigs were injected with SRBC-PEC which had been incubated in ^{131}I-γ_2-anti-SRBC antibody. The other group of recipients received separate injections of the same number of SRBC-PEC (i.p.) and comparable amounts of γ_2-anti-SRBC antibody (i.v.). The third group was immunized with the same number of SRBC injected intraperitoneally 3 hours prior to injection of PEC coated with γ_2-anti-SRBC antibody. The recipients were skin tested 7 days later.

TABLE 5

TWENTY-FOUR-HOUR SKIN REACTIONS IN GUINEA-PIGS IMMUNIZED WITH PEC-ASSOCIATED SRBC. THE EFFECT OF INJECTION OF ANTI-SRBC ANTIBODY SEPARATELY OR ON THE SAME PEC

Antigen	Mean induration	Mean diameter
PEC-SRBC+NGPS	5·1 ± 0·7	−ve
PEC-SRBC+gamma$_2$ anti-SRBC	8·4 ± 1·4	14·2+++
PEC-SRBC; gamma$_2$ anti-SRBC, i.v.	5·1 ± 0·6	−ve
PEC-gamma$_2$ anti-SRBC +SRBC	8·0 ± 1·9	10·8++
PEC+gamma$_2$ anti-SRBC; SRBC i.p.	6·0 ± 1·5	−ve
SRBC 1×10^8	3·0 ± 1·6	−ve
Nil	0·5 ± 0·5	−ve

Guinea-pigs were immunized with SRBC-PEC. Comparable amounts of ^{131}I-labelled γ_2-anti-SRBC globulin were injected on PEC or separately (intravenously). Some guinea-pigs received PEC coated with anti-SRBC γ_2-globulin and then incubated with SRBC, or SRBC were injected separately (intraperitoneally) 3 hours before. The figures correspond to 24-hour skin reaction on a challenge on day 7.

Table 5 indicates that anti-SRBC antibody only enhanced the induction of DH when it was present on the PEC. The same amount of antibody injected separately had no effect. Animals injected with SRBC before the injection of Cab-coated PEC exhibited reactions comparable to those elicited by SRBC-PEC which were incubated in NGPS but stronger than the reactions induced by 'free' SRBC.

It was concluded that the presence of anti-SRBC antibody on PEC was critical for its ability to augment the induction of DH skin reactions.

THE EFFECT OF ANTI-SRBC ANTIBODIES ON THE IMMUNOGENICITY OF SRBC-PEC FOR HUMORAL RESPONSE

The antibody (haemagglutinating, haemolytic or cytophilic) response to SRBC when studied at 8 days after antigen injection was very poor and no differences between groups were found. However, when guinea-pigs were retested with soluble erythrocyte antigen at 14 days and then bled, the humoral response was much higher (Table 6). Guinea-pigs injected with SRBC-PEC but otherwise untreated, showed higher titres of antibodies than animals injected with SRBC associated with PEC which had been treated with anti-SRBC serum. This was in contrast to their skin reactivity. The antibody response of

TABLE 6

ANTIBODY RESPONSE TO SRBC 16 DAYS AFTER IMMUNIZATION

Antigen	Antibody titre			
	Experiment 1		Experiment 2	
	Haemagglutinating	Cytophilic	Haemagglutinating	Haemolytic
PEC-SRBC+NGPS	5·0±1·2	2·7±0·8	8·0±1·1	8·0±1·4
PEC-SRBC+anti-SRBC	0·5±0·3	0	4·0±0·8	3·0±0·6
PEC-SRBC+anti-SRBC absorbed	1·7±0·8	0·5±0·3	5·0±1·6	4·5±0·8
PEC-SRBC+anti-SRBC pepsin-treated	5·5±1·4	2·8±0·6	7·0±1·2	7·3±1·1
SRBC 1×10^8	1·3±0·9	0	3·7±0·6	1·7±1·0
Nil	0	0	0·5±0·2	0
PEC+anti-SRBC+NRS +SRBC	5·7±1·0	5·3±1·3		
PEC+anti-SRBC+AGPGS +SRBC	7·6±1·1	7·7±1·1		
PEC+anti-DT+SRBC	6·8±1·8	6·7±1·3		
PEC-SRBC+anti-SRBC	3·8±0·9	2·8±0·8		
SRBC 1×10^8	2·7±1·2	0		
Nil	0	0		

Guinea-pigs immunized with either 'free' SRBC or SRBC associated with PEC which had been treated with different antisera. The animals were skin-tested on day 6 and 13 and bled on 16 days after immunization for antibody determination. Figures show mean and standard deviation of the $\log_2$ titre.

guinea-pigs immunized with free SRBC was inferior when compared to animals injected with SRBC-PEC.

It was concluded that the humoral antibody response was inversely related to the magnitude of delayed hypersensitivity skin lesions under these experimental conditions.

In vitro OBSERVATIONS

PEC, either normal or antigen loaded, and then incubated with ^{131}I-labelled γ_2-anti-SRBC antibody bound approximately 0·8–1·0 μg of antibody/10^6 cells (Table 7). The process of Cab binding was complete after 1 hour at 4° as longer incubation did not increase the cell-associated radioactivity. The amount of cell-bound antibody was dependent on its concentration in the medium but even under optimal conditions only insignificant (approx. 0·2 per cent) part of accessible antibody was bound to the cell. This corresponded roughly to about 3×10^6 antibody molecules per cell.

Preliminary experiments showed that normal PEC coated with Cab were able to form SRBC rosettes after 18–24 hours incubation in antibody-free medium at 37°. In contrast, SRBC-loaded PEC treated similarly formed rosettes up to the fifth hour of incubation only. The earlier disappearance of Cab from the SRBC-loaded PEC was confirmed in experiments with ^{131}I-labelled ultrasonicated soluble erythrocyte antigen (Fig. 1). Three groups were used: PEC coated with Cab, Cab-coated PEC which had been phagocytosed SRBC, or SRBC-PEC which were then treated with Cab. At zero time SRBC-PEC covered with Cab showed the highest binding ability. After 12 hours Cab-coated PEC still bound five times more antigen than the antigen-loaded PEC. The maximal uptake of solubilized erythrocyte antigen by Cab-coated PEC corresponded roughly to $2–3 \times 10^6$ antigen molecules per cell.

TABLE 7

THE BINDING OF ^{131}I-LABELLED γ_2-ANTI-SRBC ANTIBODY by PEC

γ_2-globulin concentration (μg/10^6 cells)	γ_2-globulin uptake (μg/10^6 cells)	
	PEC	PEC-SRBC
500	0·8	1·0
250	0·8	NT *
100	0·4	
50	0·1	
25	0·03	
10	0	

Normal PEC, or PEC which had phagocytosed SRBC, were treated with different concentrations of ^{131}I-labelled anti-SRBC γ_2-globulin for 1 hour at 4°. Cell-associated radioactivity was recorded and amounts of cell-bound protein was calculated. Each figure is a mean of triplicate determinations.

* NT = not tested.

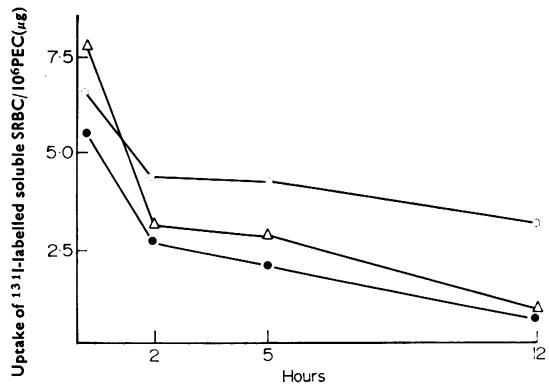

FIG. 1. Antigen-binding capacity of cytophilic antibody-coated PEC. Uptake of ^{131}I-labelled soluble erythrocyte antigen by PEC was compared within three groups: (○) PEC treated with anti-SRBC antibody without phagocytosed SRBC; (●) PEC treated with anti-SRBC antibody with phagocytosed SRBC; (△) PEC which phagocytosed SRBC and was then treated with anti-SRBC antibody.

It was concluded that antigen-binding capacity of Cab-coated PEC decreased rapidly when the cells were allowed to phagocytose SRBC prior to the addition of soluble SRBC antigen.

DISCUSSION

There is no direct evidence that the macrophage is the important cell for the antigen processing in the induction of delayed hypersensitivity. However, in some experimental systems, macrophage-associated antigen is more immunogenic than 'free' antigen (Bloch and Nordin, 1960; Asherson *et al.*, 1971; Seeger and Oppenheim, 1972). The present experiments confirm this conclusion and show that small amounts of SRBC associated with PEC induce skin reactions while the same amounts of 'free' SRBC are ineffective. SRBC-

PEC were also effective in priming for the antibody production. This is in agreement with recent observations of Seeger and Oppenheim (1972). Our experiments indicate that anti-SRBC antibody on PEC preferentially augments DH response with concomitant depression of antibody formation. This phenomenon is immunologically specific since irrelevant antibody was inactive. These observations are in keeping with findings of Liew and Parish (1972), that passively administered antibody enhances the cell-mediated immunity. Our observations, however, point to the role of macrophage Cab in the preferential induction of skin reactions. The evidence that Cab is involved in the system is as follows. (a) Anti-SRBC serum obtained 10 days after antigen injection (low Cab level) was inferior in augmentation of the immunogenicity of PEC-associated antigen to serum with high Cab titre (obtained 21 days after immunization). (b) The enhancement may be partially blocked by incubation of anti-SRBC antibody-coated PEC with anti-guinea-pig globulin serum. (c) PEC from sensitized donors, known to carry Cab (Boyden, 1964), were more active than normal PEC. (d) The serum with high titres of HA antibodies, but deprived of cytophilic activity by absorption or by destroying the Fc fragment, was inactive. (e) Iodoacetamide, which destroys the macrophage receptor for Cab (Berken and Benacerraf, 1966), prevents the augmentation caused by anti-SRBC antibody. (f) Separate injection of the same amount of anti-SRBC antibody, which is effective when on PEC, does not enhance the DH response.

The intriguing inverse relationship between DH and humoral response was also observed by Liew and Parish (1972) and is discussed in detail in their paper. There are several possible explanations of the mechanism of Cab activity. (i) Cab on macrophages limits antigen escape by covering the cell surface and thus allows antigen degradation to proceed for a longer time. Alternatively the formation of immune complexes may hold the antigen in place. The degree of degradation is relevant as Pearson and Raffel (1971) suggested that DH was favoured by a high degree of antigen degradation by macrophages while antibody production was favoured by lesser degrees of degradation. (ii) Uhr, Salvin and Pappenheimer (1957) showed that the immune complexes formed in antibody excess produced DH preferentially. Similar immune complexes may be formed on the surface of macrophages when antigen reaches the surface from the interior of the cell and combines with cytophilic antibody. Direct evidence for antigen on the macrophage surface is provided by ultrastructural studies of Unanue *et al.* (1968) and is also suggested by the present findings that attached Cab, as judged by the presence of free antigen binding sites, disappears at an early time from antigen-loaded macrophages. (iii) Cab on the macrophage surface facilitates the interaction with T lymphocytes, perhaps by rendering the antigen more hydrophobic. Seeger and Oppenheim (1972) and Kunin *et al.* (1972) suggested that macrophage-associated antigen is effective in activating T cells. In our system, however, SRBC associated with macrophages were only moderately efficient in eliciting the DH response unless coated with presumptive cytophilic antibody.

It is not clear whether cytophilic antibody involvement in the regulation of DH response is only a laboratory artifact or whether it has some significance *in vivo*. Present experiments suggest the possibility of deliberate and selective manipulation of cell-mediated immune responses. In particular, the use of purified cytophilic antibodies might facilitate *in vivo* induction of cell-mediated tumour immunity.

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