Immunology, 1974, **27**, 563.

Cytophilic Antibodies in Mice Contact-sensitized with Oxazolone

IMMUNOCHEMICAL CHARACTERIZATION AND PREFERENTIAL BINDING TO A TRYPSIN-SENSITIVE MACROPHAGE RECEPTOR

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(*Received* 6*th December* 1973; *accepted for publication* 9*th March* 1974)

Summary. Oxazolone-specific cytophilic antibodies in the sera of non-immune CBA mice and mice contact-sensitized with oxazolone, were studied with a rosette test employing peritoneal exudate macrophages and oxazolone-coupled sheep erythrocytes. Macrophage rosettes, produced by direct or indirect cytophilic antibodies, were found to depend on optimally hapten-coupled erythrocytes. Sera obtained 1 week after contact sensitization with oxazolone contained principally 7S IgG2a cytophilic antibodies. Monomeric 7S IgM antibodies cytophilic for macrophages may have been present as well. Primary contact sensitization and boosting was found uniquely to lead to high titres of hyperimmune oxazolone cytophilic antibodies predominantly binding to trypsin-sensitive macrophage receptors.

It has been previously shown that specifically sensitized macrophages mediate a component of delayed skin reactions in mice contact sensitized with oxazolone. Since these reactions can be transferred by immune serum or by normal macrophages coated with immune serum, and since acquisition of this passive sensitization can be abolished by prior trypsinization of these cells, it is suggested that 7S IgG2a and/or 7S IgM cytophilic oxazolone antibodies attaching to trypsin-sensitive macrophage receptors, mediate the specific macrophage component of contact sensitivity in mice.

INTRODUCTION

Cytophilic antibodies are serum factors which attach to cells by their Fc portion, rendering these cells capable of interacting with antigen. The exact immunological role of antibodies cytophilic for macrophages is unknown. Recent work on contact sensitivity of mice has shown that delayed reactions following contact challenge are complex phenomena composed of two distinct components (Asherson and Zembala, 1970; Zembala and Asherson, 1970). One portion of this response can be transferred with lymphocytes that are sensitive to anti-theta serum (Zembala and Asherson, 1973), and is therefore probably a classical delayed hypersensitivity reaction.

The other component of 'delayed-in-time' contact reactions of mice appears to be

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mediated by macrophages coated with cytophilic antibodies. The evidence in this system for specific macrophage-mediated immunity is: (1) transfer of skin reactions with purified macrophages (99 per cent pure) from actively sensitized donors (Asherson and Zembala, 1970); (2) inhibition of this transfer by treating actively sensitized macrophages with trypsin or anti-mouse globulin (similar treatment of sensitized lymphocytes does not abolish their transfer); (3) transfer by normal macrophages coated with serum from sensitized donors (normal lymphocytes do not similarly acquire passive sensitization); (4) transfer by immune serum to macrophage-restored, irradiated recipients (Zembala and Asherson, 1970).

These data indicate a probable role for macrophage cytophilic antibodies in contact reactions of mice. In the present study *in vitro* methods were developed for demonstrating oxazolone-specific cytophilic antibodies in the sera of mice contact-sensitized with oxazolone; and the immunochemical character of these antibodies was determined. Zembala and Asherson (1970) found that transfer of contact reactions by macrophages passively sensitized with immune serum factors, could be prevented by prior trypsinization of these cells. With the development of a rosette technique detecting cytophilic antibodies in the sera of contact-sensitized mice *in vitro*, it was important to examine whether these rosettes were mediated by antibodies attached to trypsin-sensitive macrophage receptors. The present study reports that contact sensitization and boosting of mice uniquely produces cytophilic antibodies preferentially binding to a trypsin-sensitive macrophage receptor.

MATERIALS AND METHODS

Animals and immunization

Two to four-month-old male CBA/CaJ and CBA/HT6J mice were used (Jackson Laboratories, Bar Harbor, Maine). Mice were contact-sensitized by applying 0·1 ml of 3 per cent oxazolone [2-phenyl-4-ethoxymethylene oxazolone (Gallard-Schlesinger, Carle Place, New York)], in ethanol to the skin of the clipped abdomen. In some experiments, this procedure was used for contact boosting at weekly intervals. Mice immunized with erythrocytes received 0·2-ml intraperitoneal injections of 10 per cent cells at weekly intervals. In one experiment an oxazolone–keyhole limpet haemocyanin conjugate (Ox–KLH) in phosphate-buffered saline was emulsified with Freund's complete adjuvant (FCA) (Difco, Detroit, Michigan) and injected (50 μg per mouse) in a total volume of 0·2 ml distributed among the four proximal extremities.

Serum

Pooled blood from five to thirty donors was obtained 7 days after primary immunization or boosting by severing the vessels of the neck. Serum was isolated after clotting and stored at $-90°$ in small aliquots so that samples tested for cytophilic antibodies were thawed only once.

Reagents

Blue Dextran and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey, U.S.A. Previous publications have described the preparation of rabbit anti-mouse gamma-globulin (Zembala and Asherson, 1970) and rabbit antisera monospecific for mouse IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b} and mouse light chains (Torrigiani,

1972). The specificity of these reagents has been previously determined (Greaves, 1971). Picryl-human serum albumin ($Pic_{16}HSA$) was a gift of Dr Fred S. Kantor. The preparation of Oxazolone–HSA ($Ox_{16}HSA$) has been previously described (Askenase and Asherson, 1972), as has the preparation of Ox–KLH (Askenase, 1973). To obtain RBC with varied coating densities of oxazolone, a final constant concentration (164 μg/ml) of freshly prepared reactive hapten in pH 8·4 EDTA buffer was mixed with varied concentrations of SRBC (Askenase and Asherson, 1973). In studies on anti-immunoglobulin inhibition of direct cytophilic antibodies, rabbit anti-mouse sera were absorbed with Ox–SRBC to remove natural antibodies.

Macrophages

Media consisting of Hanks's balanced salt solution (HBSS) and 1 per cent foetal calf serum were used to harvest peritoneal exudate cells (PEC) from unimmunized mice 3 days after intraperitoneal injection of 2·5 ml of Brewers thyoglycollate medium (Difco Laboratories, Detroit, Michigan). PEC were washed twice at 1000 rev/min in an International PR-2 centrifuge for 5 minutes at 2° and then 50 μl of resuspended cells were added to 0·425 ml medium containing 50 μl of 0·1 per cent Neutral Red (Fisher Scientific). After 5 minutes incubation at 23°, 25 μl of 0·4 per cent Trypan Blue (Gibco, Grand Island, New York) was added and live macrophages were enumerated in a Neubauer haemocytometer by counting those cells staining with Neutral Red and excluding Trypan Blue. Approximately $1{\cdot}5 \times 10^7$ PEC containing 90 per cent live macrophages were obtained per mouse.

Direct cytophilic antibody assay

A modification (Sulitzeanu and Haskill, 1972) of the suspension centrifugation technique was employed (Jonas, Gurner, Nelson and Coombs, 1964). The entire assay was performed at 0–2° and all glass surfaces were siliconized. A pellet containing about 5×10^5 PEC in a 10×75 mm glass tube was resuspended with 0·3 ml of undiluted serum, and incubated for 45 minutes. These passively sensitized macrophages were then washed three to four times and finally resuspended in 1 ml medium containing 0·05 mg Neutral Red and 0·1 per cent Ox–SRBC. This cell mixture was divided among three polyethylene conical micro (0·4 ml) centrifuge tubes (Beckman Instruments, Fullerton, California). These tubes were centrifuged at 1500 rev/min for 5 minutes and left overnight at 4°. The next day, the supernatant was removed and the pellet gently resuspended in plain HBSS containing 0·025 per cent glutaraldehyde. Coded triplicate samples were read at random. Neutral Red-positive cells with four or more adherent red cells were considered macrophage rosettes and the results were expressed as percentage of rosetted macrophages after scoring (100–200) unclumped macrophages.

Inhibition of macrophages rosettes formed by the direct technique was accomplished by resuspending the washed and passively sensitized PEC in hapten–protein conjugates and medium controls (Fig. 1) or rabbit anti-mouse immunoglobulin reagents (1:100) and pooled normal rabbit sera (1:100) and medium controls (Table 4). Macrophages were incubated with inhibitors for 30 minutes, washed, combined with Ox-SRBC, and divided among triplicate microcentrifuge tubes as above.

Indirect (adherence promoting) cytophilic antibody assay

Serial dilutions of sera in 1-ml volumes of medium containing 0·1 per cent Ox–SRBC

were incubated at 23° in 12×75 mm plastic tubes (Falcon Plastics, Oxnard, California) for 45 minutes. An additional incubation of 30 minutes, and all subsequent steps were performed at 0–4°. Sensitized erythrocytes were washed twice and brought to 1·0 ml with medium containing 0·05 mg of Neutral Red and 5×10^5 PEC. This cell mixture was centrifuged in triplicate in conical micro-tubes and read as in the direct assay. All cytophilic antibody tests had medium controls to identify rare instances when Ox–SRBC rosetted with macrophages in the absence of antibody, and positive rosette controls employing a high titred antisera.

The indirect cytophilic antibody titre was taken to be the highest serum dilution causing 15 per cent or greater macrophage rosettes. Inhibition of indirect cytophilic antibody tests was accomplished by performing serial dilutions of sera in medium containing Ox–HSA. Cytophilic antibody titres were unaffected by treating immune sera at 56° for 45 minutes, by six cycles of freezing and thawing, by the absence of calcium, or by treatment with 2-mercaptoethanol.

Haemagglutinations and chromatography

Passive direct haemagglutination using Ox–SRBC and class-specific, antiglobulin-augmented haemagglutination were performed by previously described methods using anti-immunoglobulins diluted 1:250 (Askenase and Asherson, 1972). Molecular size of antibodies was determined in three separate experiments by applying 4–5 ml sera to an upward flowing 5×100 cm Sephadex G-200 column previously calibrated with Blue Dextran, bovine gamma-globulin and human serum albumin. Fractions were eluted with pH 7·4, 0·01 M potassium phosphate-buffered saline (PBS). Pooled fractions were tested for haemagglutinating antibody and reconcentrated by negative pressure dialysis for direct cytophilic antibody assay.

The oxazolone specificity of antiglobulin-augmented antibodies was demonstrated by inhibition with Ox–HSA (Askenase and Asherson, 1972).

Trypsin treatment of macrophages

PEC from each mouse were harvested into separate iced 50-ml conical plastic tubes (Falcon Plastics, Oxnard, California), using plain Hanks's buffered salt solution (HBSS). After 900 rev/min centrifugation for 5 minutes at 2° the cells were combined and re-suspended in HBSS or HBSS containing 0·05 per cent trypsin (three times recrystalized, Worthington Biochemical Corporation, Freehold, New Jersey) and incubated at 23° for 30 minutes. Trypsinization was stopped by adding HBSS containing 10 per cent foetal calf sera (FCS) and the cells were washed three times and then used in the direct and indirect cytophilic antibody assays. Viability (Trypan Blue exclusion) was greater than 75 per cent after trypsinization.

RESULTS

INFLUENCE OF ERYTHROCYTE–HAPTEN DENSITY ON MACROPHAGE ROSETTES MEDIATED BY CYTOPHILIC ANTIBODIES

Sera from non-immune and contact-sensitized mice were tested for titres of cytophilic antibodies using oxazolone-coated sheep erythrocytes (Ox–SRBC) and thioglycollate-induced peritoneal exudate macrophages. Red cells of increasing hapten densities were obtained by interacting decreasing concentrations of SRBC with a constant concentration

of reactive oxozolone. Table 1 (third column) shows that indirect cytophilic antibody titres increased progressively as the hapten density of oxazolone-coated erythrocytes increased, until a plateau was reached. At still higher ratios of Ox-RBC coupling, titres were decreased. The second column of Table 1 shows that when highly coated Ox-SRBC were employed a cytophilic factor in non-immune mouse sera was detected by the indirect technique.

The relationship between the hapten substitution of erythrocytes and the number of rosettes formed was similarly investigated using the direct technique. Cytophilic antibodies

TABLE 1

DIRECT AND INDIRECT CYTOPHILIC ANTIBODY TESTS USING VARIOUS OXAZOLONE-COATED SHEEP ERYTHROCYTES

SRBC concentration used for coupling* (per cent)	Indirect cytophilic antibody titre		Direct cytophilic antibody assay	
	Non-immune mouse sera	Oxazolone immune sera	Macrophages from mice actively contact-sensitized with oxazolone (per cent rosettes)	Macrophages passively sensitized with serum from oxazolone contact-sensitized mice (per cent rosettes)
80	1:5	1:250	0	5
40	1:5	1:8000	10	10
20	1:5	1:32,000	20	20
10	1:50	1:32,000	25	40
5	1:50	1:32,000	0	15
2·5	n.t.†	1:8000	0	5

* The various SRBC concentrations were coupled by exposure to a constant concentration (164 μg/ml) of reactive oxazolone.
† n.t. = Not tested.

directly coating macrophages were measured by first incubating serum from contact-sensitized mice with the macrophages. These passively sensitized cells were then washed and finally centrifuged together with Ox–SRBC coupled with various hapten densities. The last column of Table 1 shows that rosettes produced by the direct technique also rose to an optimum with increasing oxazolone coating of the erythrocytes. In addition, cytophilic antibodies detected on peritoneal exudate macrophages of actively sensitized mice (Table 1, Column 4) showed the same optimal formation of rosettes with moderately coupled red cells that was found with serum antibody by the direct and indirect techniques. It was concluded that cytophilic antibodies detected in these three instances were similar in hapten density requirements of indicator erythrocytes. In subsequent experiments coupling with oxazolone was performed at the optimal SRBC concentration of 10 per cent.

SPECIFICITY OF CYTOPHILIC ANTIBODIES

The antigenic specificity of cytophilic antibodies was determined using both the direct and indirect methods. In the direct assay, aliquots of passively sensitized macrophages were added to tubes containing serial ten-fold dilutions of inhibiting Ox_{16}HSA or Pic_{16} HSA. After incubation, these cells were washed and finally centrifuged together with Ox–SRBC. Fig. 1 shows that rosettes obtained were strongly inhibited by prior incubation

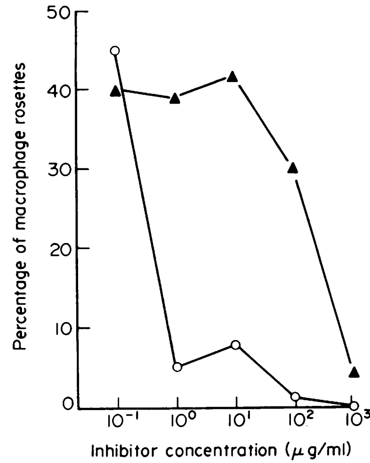


FIG. 1. Specificity of direct cytophilic antibodies in the sera of CBA mice 1 week after oxazolone contact sensitization, determined by incubating passively coated macrophages in various concentrations of of $Ox_{16}HSA$ (○) and picryl$_{16}$HSA (▲) prior to rosetting with Ox–SRBC.

TABLE 2

SPECIFICITY OF INDIRECT CYTOPHILIC ANTIBODY ASSAY USING HYPER-IMMUNE SERUM FROM OXAZOLONE CONTACT-SENSITIZED MICE

Serum dilution	Serum dilutions in plain buffer, then titration with Ox–SRBC (per cent rosettes)	Serum dilutions in Ox–HSA, then titration with Ox–SRBC (per cent rosettes)
1:10,000	75	5
1:20,000	65	5
1:40,000	70	0
1:80,000	35	0
1:160,000	10	0
1:320,000	5	0
1:640,000	0	0

TABLE 3

SPECIFICITY OF INDIRECT CYTOPHILIC ANTIBODY ASSAY USING SERUM FROM NON-IMMUNE CBA/CaJ MICE

Serum dilution	Serum dilutions in plain buffer, then titration with Ox–SRBC (per cent rosettes)	Serum dilutions in $Ox_{16}HSA$, then titration with Ox–SRBC (per cent rosettes)
1:5	40	5
1:10	20	5
1:20	15	5
1:40	15	10
1:80	15	10
1:160	5	5
1:320	0	0

of antibody-coated macrophages in Ox–HSA concentrations of 1 μg/ml and greater, while Pic-HSA was weakly cross-reactive by inhibiting rosettes at 1000 μg/ml.

To test immunological specificity of macrophage rosettes by the indirect method, serum from oxazolone contact-sensitized mice was serially diluted in medium containing Ox–HSA. Table 2 shows that this caused complete inhibition, of rosettes mediated by cytophilic antibodies in immune sera. In addition, factors in non-immune sera which caused Ox–SRBC to form rosettes by the indirect technique, were also inhibited by prior incubation in Ox–HSA (Table 3). It was concluded that both the direct and indirect techniques detected oxazolone-specific cytophilic antibodies in the sera of contact-sensitized mice and that small amounts of 'natural' oxazolone-specific cytophilic antibodies were present in non-immune mouse sera.

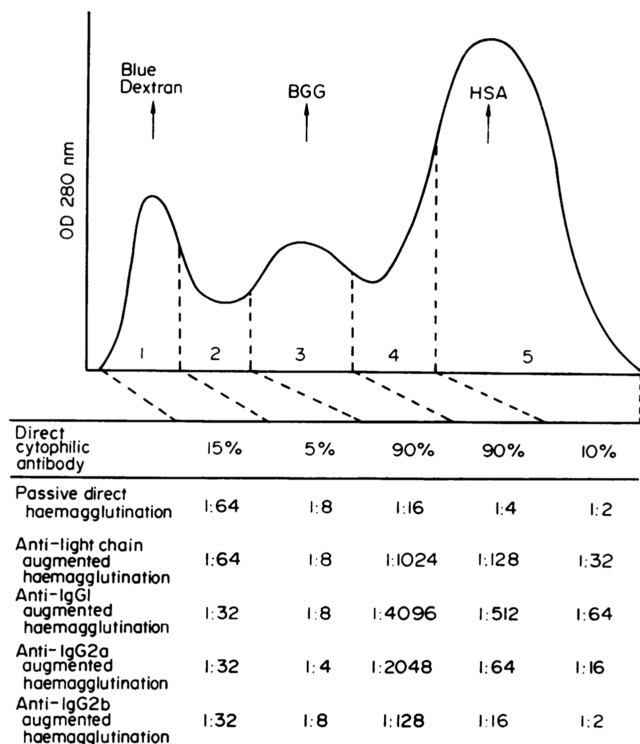
ELUTION OF DIRECT CYTOPHILIC ANTIBODIES BY WASHING AND BY 56°

Washing passively sensitized macrophages in the direct method was found to elute absorbed antibodies and exhaustive washing removed all detectable cytophilic antibodies from the cell surface. With incomplete washing of passively sensitized macrophages, eluted antibodies in the wash fluid contributed to direct cytophilic antibody determinations by acting as opsonins when the red cells were later added. Thus, when washing was inadequate, direct rosettes were in part really indirect rosettes. Elution was detected by performing indirect assays on wash fluids from the direct assay. Cytophilic antibodies were present in excess of those expected from dilution of antibodies originally failing to bind to macrophages. To detect antibodies truly adhering to macrophages in the absence of antigen, the adequacy of direct cytophilic antibody washes was assured by performing indirect cytophilic antibody tests on the final direct assay wash fluid prior to adding Ox–SRBC.

With adequate precautions to exclude antibodies eluted by washing, treatment of antibody-coated macrophages at 56° for 30 minutes resulted in additional elution of cytophilic antibodies. Because cells treated at 56° failed to attach cytophilic antibodies by the direct or indirect tests, elution of cytophilic antibodies at 56° may have been due to destruction of receptors for cytophilic antibodies. Treatment at 56° of macrophages from actively sensitized mice produced similar elution of cytophilic antibodies.

MOLECULAR SIZE OF CYTOPHILIC ANTIBODIES

Serum from oxazolone contact-sensitized mice was passed through a calibrated column of Sephadex G-200. Eluted fractions were assayed for antibody by passive direct and antiglobulin-augmented haemagglutination which gave similar results in three experiments. Pools of appropriate fractions were concentrated and assayed for direct cytophilic antibodies. Fig. 2 shows that direct haemagglutinating antibody was found principally in excluded (19S) fractions while anti-light chain-augmented haemagglutination demonstrated substantial titres in 7S fractions as well. Augmented haemagglutination with class-specific reagents showed that the classes of 7S antibodies were mainly IgG1 and IgG2a with some IgG2b as well. There was no augmentation with anti-IgM or anti-IgA. The chromatogram in Fig. 2 is from an experiment in which direct cytophilic assay of reconcentrated pooled fractions showed that almost all cytophilic antibodies were found in the 7S factions as depicted.



	1	2	3	4	5
Direct cytophilic antibody	15%	5%	90%	90%	10%
Passive direct haemagglutination	1:64	1:8	1:16	1:4	1:2
Anti-light chain augmented haemagglutination	1:64	1:8	1:1024	1:128	1:32
Anti-IgG1 augmented haemagglutination	1:32	1:8	1:4096	1:512	1:64
Anti-IgG2a augmented haemagglutination	1:32	1:4	1:2048	1:64	1:16
Anti-IgG2b augmented haemagglutination	1:32	1:8	1:128	1:16	1:2

FIG. 2. Fractionation of sera from CBA mice 1 week after oxazolone contact-sensitization, on a 5 × 100 cm Sephadex G-200 column previously calibrated with Blue Dextran, bovine gamma-globulin (BGG) and human serum albumin (HSA). Passive direct haemagglutination and class-specific antiglobulin augmented haemagglutination titres were obtained using fractions directly from the column, while direct cytophilic antibody assay was performed using pooled and reconcentrated fractions.

TABLE 4

ANTIGLOBULIN INHIBITION OF DIRECT CYTOPHILIC ANTIBODIES IN THE SERA OF MICE 1 WEEK AFTER CONTACT SENSITIZATION WITH OXAZOLONE

Inhibiting rabbit anti-mouse immunoglobulin*	Percentage rosette inhibition ± s.d.†
Anti-light chains	100 ± 2
Anti-mouse gamma-globulin	85 ± 6
Anti-IgG2a	95 ± 4
Anti-IgM	55 ± 10
Anti-IgG2b	20 ± 12
Anti-IgA	20 ± 7
Anti-IgG1	10 ± 7

* Inhibiting antiglobulins were heated at 56° for 30 minutes, absorbed with Ox–SRBC to remove natural antibodies, and used at a dilution of 1 : 100.

† Figures are given as percentages of control samples treated with 1 : 100 normal rabbit serum. The percentages represent mean values (± standard deviation of the mean) for a series of six to eight experiments.

IMMUNOGLOBULIN CLASS OF DIRECT CYTOPHILIC ANTIBODIES IN THE SERA OF OXAZOLONE CONTACT-SENSITIZED MICE

Aliquots of macrophages passively sensitized with whole immune serum were added to tubes containing 1:100 dilutions of various rabbit anti-mouse immunoglobulin reagents or to controls containing similarly diluted normal rabbit serum. After incubation with inhibitors, these cells were washed and finally centrifuged together with Ox–SRBC. The percentage of rosettes obtained after incubation with normal rabbit serum was compared with the percentage of rosettes obtained after incubation with an anti-immunoglobulin inhibitor. Thus 80 per cent rosettes after normal rabbit serum and 40 per cent rosettes after an antiglobulin was considered a 50 per cent inhibition. Data pooled from eight experiments showed that rabbit antisera containing antibodies against mouse light chains, mouse gamma-globulins, and mouse IgG2a produced. a nearly complete inhibition of direct cytophilic antibodies (Table 4). These three antiglobulins also gave substantial rosette inhibition when diluted 1:1000. Anti-IgG1, anti-IgG2b and anti-IgA were weakly inhibitory at 1:100, while there was a mean inhibition of 55 per cent in six experiments with anti-IgM. It was concluded that the principal direct cytophilic antibody in the sera of mice 1 week after contact sensitization with oxazolone was 7S IgG2a in class. Since gel filtration studies demonstrated that almost all direct cytophilic antibodies were 7S in size, and anti-IgM gave 55 per cent rosette inhibition, monomeric IgM cytophilic antibodies may have been present as well.

BINDING OF EARLY CYTOPHILIC ANTIBODIES TO TRYPSINIZED MACROPHAGES

PEC macrophages were treated with trypsin, washed and then used in cytophilic antibody tests. The sera of mice 1 week after primary contact sensitization, contained

TABLE 5

EFFECT OF MACROPHAGE TRYPSINIZATION ON INDIRECT CYTOPHILIC ANTIBODY TITRES OF PRIMARY AND BOOSTED SERA FROM MICE IMMUNIZED WITH SRBC OR OXAZOLONE CONJUGATES

	Indirect cytophilic antibody titres			
Immunization	Mouse strain	Control macrophages	Trypsinized macrophages	Change in titre with trypsinization
Primary immunization with Ox-KLH in FCA*	C3H/HeJ	1:10,240	1:640	16-fold decrease
Primary immunization with Ox-HuRBC i.p.*§	C3H/HeJ	1:2560	1:640	4-fold decrease
Weekly boosting with SRBC i.p.†	CBA/H	1:160,000	1:160,000	No change
Weekly boosting with Ox-HuRBC i.p.‡§	CBA/CaJ	1:256,000	1:256,000	No change

* Sera obtained 1 week after immunization.
† Sera obtained 14 weeks after initial immunization.
‡ Sera obtained 6 weeks after initial immunization.
§ Oxazolone–human RBC.

cytophilic antibodies which were inhibited in binding to trypsinized macrophages. Table 5 shows that predominant binding to trypsin-sensitive macrophage receptors was also a property of mouse cytophilic antibodies obtained 1 week after primary immunization with Ox–KLH in FCA or 1 week after intraperitoneal injection of Ox–HuRBC. However,

boosted sera from mice receiving multiple intraperitoneal injections of SRBC or Ox–HuRBC had anti-sheep cell and anti-oxazolone cytophilic antibody titres that were unaffected by macrophage trypsinization (Table 5). It was concluded that primary immunization by a variety of means resulted in early occurring cytophilic antibodies preferentially attaching to trypsin sensitive macrophage receptors.

TABLE 6

EFFECT OF MACROPHAGE TRYPSINIZATION ON INDIRECT CYTOPHILIC ANTIBODY TITRES OF SERA FROM OXAZOLONE CONTACT-SENSITIZED MICE AFTER PRIMARY OR BOOSTED IMMUNIZATION

	Indirect cytophilic antibody titre			
Immunization	Mouse strain	Control macrophages	Trypsinized macrophages	Change in titre with trypsinization
Primary contact-sensitization*	CBA/HT6J	1:25,600	1:6400	4-fold decrease
Primary contact-sensitilization*	CBH/CaJ	1:4000	1:250	16-fold decrease
Primary contact-sensitization*	C3H/HeJ	1:4000	1:800	5-fold decrease
Boosted contact-sensitization†	CBA/HT6J	1:512,000	1:128,000	4-fold decrease
Boosted contact-sensitization†	CBA/CaJ	1:160,000	1:20,000	8-fold decrease
Boosted contact-sensitization†	C3H/HeJ	1:40,960	1:5120	8-fold decrease

* Sera obtained 1 week after sensitization.
† Sera obtained 3–4 weeks after initial sensitization.

TABLE 7

EFFECT OF MACROPHAGE TRYPSINIZATION ON DIRECT CYTOPHILIC ANTIBODY TITRES OF SERA FROM OXAZOLONE CONTACT-SENSITIZED MICE AFTER PRIMARY OR BOOSTED IMMUNIZATION

Immunization	Mouse strain	Control macrophages	Trypsinized macrophages	Change in titre with trypsinization
Primary contact-sensitization*	CBA/HT6J	1:128	1:16	8-fold decrease
Primary contact-sensitization*	CBA/CaJ	1:64	1:8	8-fold decrease
Boosted contact-sensitization†	CBA/CaJ	1:1024	1:64	16-fold decrease

* Sera obtained 1 week after sensitization.
† Sera obtained 3 weeks after initial sensitization.

THE ATTACHMENT TO TRYPSINIZED MACROPHAGES OF CYTOPHILIC ANTIBODIES IN THE SERA OF MICE RECEIVING PRIMARY OR BOOSTED CONTACT SENSITIZATION

It was confirmed in several strains of mice that sera obtained 1 week after oxazolone contact sensitization contained cytophilic antibodies attaching to trypsin-sensitive receptors (Table 6). When these animals were additionally boosted with two, three, or four weekly oxazolone contact paintings, higher titres of cytophilic antibodies were obtained which still preferentially attached to macrophage receptors sensitive to trypsin treatment. It was

concluded that primary or boosted contact sensitization resulted in early or late serum cytophilic antibodies predominantly attaching to trypsin-sensitive macrophage receptors, while boosting by other means resulted in late cytophilic antibodies without this property.

The finding that cytophilic antibodies in the sera of mice following primary or boosted contact sensitization, attached to trypsin-sensitive receptors; while other forms of immunization resulted in primary but not boosted cytophilic antibodies attaching to trypsin-sensitive receptors, might have been peculiar to the indirect technique used. However, when these sera were employed in the direct assay, primary and boosted sera from contact-sensitized mice similarly contained early and late occurring cytophilic antibodies binding to trypsin-sensitive receptors (Table 7).

DISCUSSION

This report is the first study characterizing cytophilic antibodies in contact sensitivity. Rosettes mediated by passively or actively coated macrophages, or by antibodies initially coating red cells before adhering to normal macrophages, were all found to depend on erythrocytes optimally coupled with hapten. Thus no differences were observed between direct and indirect (adherence promoting) cytophilic antibodies in their hapten density requirements of indicator erythrocytes. Direct cytophilic antibodies were easily eluted from actively and passively sensitized macrophages by washing, and were demonstrated to be indirect cytophilic antibodies. Elution was shown to be enhanced by 56° treatment of the macrophages which may have destroyed receptors for cytophilic antibodies. Berken and Benacerraf (1966) concluded that direct and indirect cytophilic antibodies were identical and because direct cytophilic antibodies were easily removed from macrophages, Benacerraf (1968) felt that the major *in vivo* role of cytophilic antibodies was initially to act as opsonins and thus attach antigen to macrophages in the form of immune complexes. The results of the present study are entirely consistent with these assertions. However, it cannot be ruled out that the weaker initial binding of direct cytophilic antibody to macrophages triggers important and distinctive cellular processes when antigen is subsequently bound or that a biologically significant subpopulation of direct cytophilic antibodies with a strong binding affinity may coat macrophages at a level below rosette assay detection limits.

In a previous report from this laboratory haemagglutination tests showed that oxazolone-specific antibodies occur in normal mouse serum (Askenase and Asherson, 1972). In the current study it was further demonstrated that some of these 'natural' oxazolone antibodies are cytophilic for macrophages. The role of natural cytophilic antibodies in immune responses is unknown. These substances may aid in antigen localization at macrophage surfaces as a prelude to macrophage processing (Unanue and Cerottini, 1970) or may facilitate macrophage–lymphocyte interactions (Feldman, 1972).

Direct and antiglobulin-augmented haemagglutination had previously shown that sera of mice contact-sensitized with oxazolone contained antibodies in several immunoglobulin classes (Askenase and Asherson, 1972). The present study was conducted to determine which of these classes of oxazolone antibodies was important in those aspects of contact sensitivity which are due to serum and to macrophages coated with cytophilic antibodies. In previous studies, various investigators (Nelson, Kossard and Cox, 1967; Lokaj, 1968; Hoy and Nelson, 1969; Tizard, 1969; Nelson, 1970; Brown and Carpenter, 1971) employing 2-mercaptoethanol treatment and fractionation of sera by size and charge, have

shown that cytophilic antibodies in mice immunized with sheep erythrocytes or sarcoma I tumour cells are IgM, IgG2 and novel alpha-2 globulins. In the current study Sephadex G-200 was used to fractionate immune sera capable of transferring contact reactions of mice (Fig. 2). Almost all oxazolone-specific cytophilic antibodies were found in 7S fractions. Additional experiments were performed employing class-specific anti-immunoglobulin reagents to inhibit passively sensitized macrophages. These studies did not require any fractionation of immune sera and showed that most cytophilic antibodies in the sera of contact-sensitized mice were IgG2a in class (Table 4).

Recently published studies have suggested that antigen interaction with thymus-derived lymphocytes may depend on monomeric 7S IgM in the surface membrane of these T cells (Hammerling and Rajensky, 1971; Marchalonis, Cone and Atwell, 1972; Hogg and Greaves, 1972). Although it is still uncertain whether such antigen-specific surface components are made by T cells, or specifically trigger these cells, it is noteworthy to the present investigations that 7S IgM derived from 19S guinea-pig serum IgM or monomeric IgM isolated from the surface of putative mouse T cells, has been shown to be cytophilic for the macrophages of each appropriate species (Rhodes, 1973; Feldman, Cone and Marchalonis, 1973). It has been suggested that macrophages may act as intermediates for T- and B-cell collaboration via such T cell-derived 7S IgM antibodies cytophilic for macrophage surface receptors (Feldman, 1972). Results of the current investigation have clearly demonstrated that the sera of mice that are capable of transferring oxazolone contact reactions, principally contain oxazolone-specific 7S IgG2a antibodies cytophilic for macrophages. Of interest was the finding that 7S IgM antibodies cytophilic for macrophages may also be present, although in obviously smaller amounts. Whether one or both of these classes of cytophilic antibody are responsible for the specific macrophage component of 'delayed-in-time' reactions in contact-sensitized and challenged mice, has yet to be determined.

The finding of 95 per cent rosette inhibition with anti-IgG2a and 55 per cent inhibition with anti-IgM (Table 4) is difficult to explain, since the specificity of these reagents has been demonstrated (Greaves, 1971) and confirmed in similar rosette inhibition studies of antigen-binding lymphocytes (Hogg and Greaves, 1972). The necessity for co-operative binding by different classes of specific cytophilic antibodies is one explanation; another possibility is that one of these antiglobulins has appropriately bound to non-oxazolone specific immunoglobulins on the macrophage surface and thereby sterically hindered rosette formation mediated by cytophilic antibodies of another class. This steric hindrance is in agreement with rosette inhibition studies of T cells, where anti-IgM and anti-theta sera both gave 85 per cent inhibition (Hogg and Greaves, 1972).

The current study shows that sera obtained from contact-sensitized mice after primary or boosted immunization, contain cytophilic antibodies whose binding is significantly reduced by macrophage trypsinization. Although trypsin treatment may alter antibody binding to macrophages without directly affecting specific surface receptors for cytophilic antibodies, it seems likely that trypsinization interferes with antibody attachment by proteolytic digestion of these receptors. The fact that transfer of contact reactions by normal macrophages coated with serum from contact-sensitized mice can be abolished by trypsinization of the macrophages prior to attachment of the serum factors (Zembala and Asherson, 1970) and the current finding that macrophage cytophilic antibodies in these sera attach to trypsin-sensitive macrophage surface receptors, suggest that the rosette assay is detecting cytophilic factors involved in the transfer of contact reactions.

Previous studies have shown that the effect of macrophage trypsinization on cytophilic antibody binding varies according to species, time after immunization and class of cytophilic antibody. The most extensive studies on the influence of macrophage trypsinization on mouse cytophilic antibody binding were performed by Nelson *et al.* (1967) and Tizard (1969) who immunized mice with sheep red cells alone or emulsified with Freund's complete or incomplete adjuvant. In additional studies, Hoy and Nelson (1969) produced cytophilic alloantibodies by giving C57Bl/6J mice multiple intraperitoneal injections of A/J sarcoma I ascites tumour cells. These investigators found macrophage cytophilic antibodies in several immunoglobulin classes and demonstrated that cytophilic antibodies arising early (7 days) after primary immunization attached to trypsin-sensitive receptors, while boosted titres of cytophilic antibodies in hyperimmune sera were not diminished in macrophage binding by macrophage trypsinzation. Studies in mice by Tizard (1971) have suggested that macrophages have multiple types of receptors for cytophilic antibodies, and Levenson, Braude and Chernokhvostova (1970) demonstrated that antigen binding to mouse macrophages via cytophilic antibodies results in surface fixation alone when antibodies arising early after immunization are employed, while cytophilic antibodies produced in hyperimmunized animals induced both adherence and subsequent ingestion.

In the current study sera were obtained from mice 1 week after immunization with oxazolone in several forms: (1) contact sensitization; (2) injection of Ox–KLH conjugates emulsified with Freund's complete adjuvant; and (3) immunization by intraperitoneal injection of Ox–HuRBC. All of these early sera contained oxazolone-specific cytophilic antibodies predominantly attaching to a trypsin-sensitive macrophage receptor. In further experiments, mice primed with intraperitoneal Ox-HuRBC and boosted with this immunogen produced increased titres of hapten-specific cytophilic antibodies which did not attach to trypsin-sensitive macrophage receptors. It seemed that these studies on oxazolone cytophilic antibodies confirmed previous reports which showed that early cytophilic antibodies attached to trypsin-sensitive receptors while boosted, late antibodies did not. However, when mice primed by oxazolone contact sensitization were boosted by weekly contact paintings, high titred cytophilic antibodies were obtained which still predominantly attached to trypsin-sensitive macrophage receptors. Data from the current study suggest that contact sensitization and boosting uniquely results in high titres of macrophage cytophilic antibodies attaching to trypsin-sensitive macrophage receptors. Since early sera from contact-sensitized mice contain predominantly 7S IgG2a and/or 7S IgM oxazolone-specific cytophilic antibodies attaching to trypsin-sensitive macrophage receptors, it is likely that contact boosting results in cytophilic antibodies of a similar nature, attaching to a similar receptor. This suggests that serum factors mediating the macrophage component of contact reactions of mice can now be purified from high titred sera to determine the exact nature of these factors and the mechanism of their role in this reaction.

ACKNOWLEDGMENTS

The authors acknowledge the advice of Dr Geoffrey L. Asherson and Dr D. Sulitzeanu in the early phases of this work, and wish to thank Dr Fred S. Kantor for his helpful criticisms in reviewing this manuscript.

This work was supported in part by grants from the American Cancer Society (IC-91), The Connecticut Division of the American Cancer Society, The Connecticut Heart

Association (grant number 515), USPHS grant number AI-11077, and The Goldhirsch Foundation.

One of us (P.W.A.) was an Allergic Diseases Academic Awardee, number AI-170829-01.

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