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Contact Sensitivity to Oxazolone in the Mouse

VIII. DEMONSTRATION OF SEVERAL CLASSES OF ANTIBODY IN THE SERA OF CONTACT SENSITIZED AND UNIMMUNIZED MICE BY A SIMPLIFIED ANTIGLOBULIN ASSAY

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Summary. CBA mouse antibodies to oxazolone were assayed by direct and antiglobulin augmented haemagglutination, using oxazolone coupled sheep erythrocytes. The antiglobulin Coombs' test was simplified by eliminating washing the sensitized cells and running the entire assay in a microtitre plate. Oxazolone antibodies of IgM, IgG_1 and IgG_{2a} classes were found in unimmunized mice, and antibodies of significantly higher titre were found in IgM, IgG_1, IgG_{2a} and IgG_{2b} classes of contact sensitized mice. Because the demonstration of oxazolone antibodies in the sera of unimmunized mice required highly oxazolonated SRBC, it was suggested that these antibodies are of relatively low binding avidity.

INTRODUCTION

Mice painted with 2-phenyl-4-ethoxymethylene oxazolone (oxazolone) develop contact sensitivity as shown by ear swelling when challenged 7 days later (Asherson and Ptak, 1968). This is a mixed reaction with humoral and cellular components. The evidence for antibody is the occurrence of ear swelling 4 hours after challenge, the presence of polymorphs in the lesions (de Sousa and Parrott, 1969), and transfer of at least part of the reaction by immune serum and macrophages coated with immune serum (Zembala and Asherson, 1970). The evidence for a classical delayed hypersensitivity component is the passive transfer with purified peritoneal exudate lymphocytes and lymph node lymphocytes (Ptak and Asherson, 1969; Asherson and Zembala, 1970).

The development of techniques for assaying antibodies to oxazolone is a necessary preliminary to investigating the mechanism of the serum mediated reactions. This report describes the measurement of mouse haemagglutinating oxazolone antibodies using oxazolone coupled to sheep erythrocytes (Ox-SRBC), and a simplified microtitre adaptation of the antiglobulin test.

MATERIALS AND METHODS

Animals and immunization (*sensitization*)

Two to 3-month old male or female CBA mice were used. They were of either

strain CBA/H obtained from Animal Service Laboratories or CBA/J from Jackson Laboratories (Bar Harbor, Maine, U.S.A.). Mice were immunized by applying 0·1 ml of 3 per cent oxazolone (2-phenyl-4-ethoxymethylene oxazolone, British Drug Houses, Ltd, Poole, Dorset, England) in ethyl alcohol to the skin of the clipped abdomen. In one experiment mice were sensitized with 0·1 ml 7 per cent picryl chloride in ethyl alcohol. Sensitized and unimmunized controls were injected with 2–3 ml of thioglycolate broth by the intraperitoneal route 4 days prior to being bled.

Serum

Pooled blood from five to twenty donors of the same sex was obtained 7 days after immunization by severing the vessels of the neck. After clotting, serum was isolated and stored at −55° or with 0·02 per cent azide at 4° after absorption with SRBC.

Reagents

Sephadex G-200 and G-25 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The preparation of rabbit anti-mouse gamma globulin has previously been described (Zembala and Asherson, 1970). Rabbit antisera prepared against mouse myeloma proteins and made mono-specific for mouse IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b} and mouse light chains by fluid phase absorption, were a generous gift of Dr G. Torrigiani. These reagents were specific on immunoelectrophoresis and Ouchterlony analysis. Precipitin in gel assay of Sephadex G-200 fractions of mouse sera showed that the rabbit antisera detected IgM, IgA and IgG where expected; and on antiglobulin testing the excluded (IgM) fractions were augmented only by anti-light chains and anti-mouse γ-globulin and not by anti-IgG reagents, while 7S fractions were not augmented by anti-IgM, but were augmented by the anti-IgG reagents. The specificity of these antisera has also been tested by immunofluorescence of immunoglobulin-producing tumours (Greaves, 1971).

Human serum albumin (HSA) and rabbit anti-whole mouse sera were obtained from Behringwerke (Hoechst Pharmaceuticals, Hounslow, Middlesex, England). Pooled normal rabbit sera (NRS) and fresh SRBC in Alsever's solution were obtained from Wellcome Research Laboratories, Beckenham, Kent, England. All rabbit antisera were inactivated and had sheep cell agglutinins removed by absorption.

Buffers

Phosphate buffered saline (PBS) was 0·01 M potassium phosphate buffer pH 7·4 with 0·15 M NaCl and 0·02 per cent sodium azide. Disodium ethylenediaminetetraacetate (EDTA) buffer pH 8·4 and EDTA-saline buffer pH 7·5 have been described (Ling, 1961).

Oxazolone coupling to SRBC

In the standard method, 0·25 ml of 1 per cent oxazolone in ethanol was squirted using a 1-cc syringe and 27-gauge needle, into 10 ml of pH 8·4 EDTA buffer on a magnetic stirrer. The aqueous oxazolone was *immediately* added, with mixing, to 5 ml of 15 per cent washed SRBC in PBS. The cells were then gently agitated for 30 minutes at room temperature. Delay in combining the aqueous exazolone and SRBC produced inferior coupling. The reaction was stopped by adding 4 volumes of iced pH 7·5 EDTA-saline; then the cells were washed three times and stored at 4° as a 5 per cent suspension in PBS.

Ox-SRBC could be used for a week without loss of titre. In some experiments (Figs 1 and 2) oxazolonation was varied by reacting different cell concentrations.

Oxozolone coupling to HSA

0·75 ml of 1 per cent oxazolone in ethanol was squirted into 25 ml of 5 mg/ml HSA in pH 8·4 EDTA buffer on a magnetic stirrer. After stirring for 60 minutes at room temperature and 30 minutes at 4°, the oxazolonated protein was freed of uncombined hapten and EDTA on a Sephadex G-25 column equilibrated with PBS at 4°. There were sixteen groups per molecule of HSA, based on the O.D. of the conjugate at 352 mμ and the data of Yoshimura and Cinader (1966). $Picryl_{16}HSA$ was a gift from Dr Fred S. Kantor.

Direct haemagglutination technique

A 1:16 dilution of absorbed mouse sera or undiluted column fraction was serially diluted two-fold in 0·025 ml of PBS containing 2·5 per cent NRS in 'V' bottom microtitre plates (Cooke Engineering Co., Alexandria, Va., U.S.A.). A 0·025-ml drop of 0·1 per cent oxazolonated SRBC in diluent was then added and the plates incubated at room temperature for 1–2 hours and at 4° overnight, before being read. The last well showing agglutination was taken as the end point. Minimal (1+) agglutinations were confirmed by low power microscopic examination using a 'blind' observer or by parallel titration in diluent containing inhibiting Ox-HSA at 0·3 mg/ml. Titres were expressed as negative logarithms of two-fold serial dilution steps (1:16 is 4).

Standard antiglobulin test

In the standard Coombs' assay the starting dilution of mouse sera was 2^6, 0·4 ml volumes were diluted two-fold in tubes, and 0·4 ml of 0·5 per cent Ox-SRBC were added. After incubation, the cells were washed and resuspended in 0·4 ml of diluent. Then, 0·025 ml were added to an equal volume of diluted antiglobulin reagent (or plain diluent to show direct haemagglutination) in microtitre wells, and the plates incubated and read as above.

Simplified antiglobulin test

The entire assay was performed in a microtitre plate without washing the sensitized cells (see Results).

RESULTS

DIRECT HAEMAGGLUTINATING ANTIBODY

Sheep red blood cells were coated with oxazolone and used to measure haemagglutinating antibody to oxazolone. Pooled sera from CBA mice 7 days after immunization gave a haemagglutination titre of 2^7–2^9, while pooled serum from unimmunized mice and serum from mice 7 days after immunization with picryl chloride had no detectable antibody (less than 2^4).

In an attempt to raise assay sensitivity by using more highly coupled cells, a constant amount of aqueous oxazolone was mixed with decreasing concentrations of SRBC. Fig. 1 shows that this resulted in a progressive rise in the titre of pooled oxazolone-immune sera and that titres obtained with lightly coated cells were inhibited by serially diluting in low concentrations of Ox-HSA (Fig. 1a), while titres with highly coated cells could only

be inhibited by high concentrations of Ox-HSA (Fig. 1d). Fig. 1 also shows that highly coated cells were agglutinated by pooled sera from unimmunized CBA mice. The haemagglutinating factors in the sera of unimmunized mice behaved like antibody as judged by inhibition with oxazolonated protein and augmentation by antiglobulin reagents (Figs 2 and 3). The agglutination of lightly coated cells with oxazolone immune sera and the agglutination of heavily coated cells with sera from unimmunized mice was shown to be immunologically specific by parallel inhibition with equal concentrations of $Ox_{16}HSA$

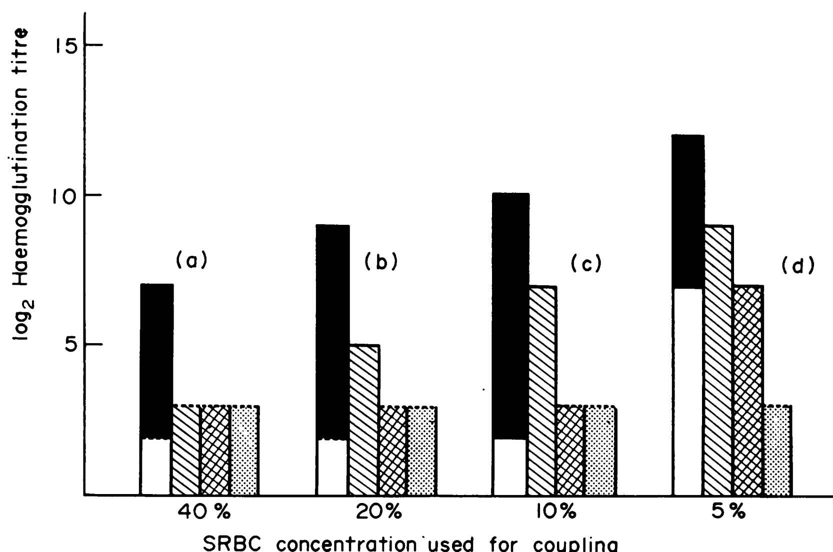

FIG. 1. Haemagglutinin titres of oxazolone-immune and non-immune sera using variously coated Ox-SRBC and different concentrations of free inhibitors. The degree of erythrocyte coating with oxazolone was varied by mixing a final oxazolone concentration of 0·163 mg/ml with the following initial SRBC suspension concentrations: (a) 40 per cent, (b) 20 per cent, (c) 10 per cent, (d) 5 per cent. Dotted lines indicate that the titre was below the starting dilution. (Open bar) sera from unimmunized CBA mice; (solid bars) HSA 3·0 mg/ml; diagonally hatched bars, OX-HSA 0·03 mg/ml; diagonally cross-hatched bars, OX-HSA 0·3 mg/ml; stippled bars, OX-HSA 3·0 mg/ml.

and $Picryl_{16}HSA$ over a wide range of equal concentrations (Fig. 2). It was concluded that there are oxazolone antibodies in the sera of unimmunized CBA mice which can be detected with highly oxazolonated cells. In subsequent experiments employing the Coombs' technique, 15 per cent cells were used for coupling.

STANDARD AND SIMPLIFIED ANTIGLOBULIN ASSAY

In a standard antiglobulin test cells are added to tubes containing serial dilutions of antisera. After incubation the sensitized cells are washed and resuspended in antiglobulin reagent causing agglutination. Similar results were obtained by antiglobulin augmented haemagglutination whether the sensitized cells were allowed to settle and were then merely resuspended in antiglobulin reagent, or when they were washed several times. This suggested that the antiglobulin test could be simplified by eliminating washing.

In the simplified assay pooled immune serum was serially diluted in a microtitre plate and 0·025 ml of Ox-SRBC in a dilution of antiglobulin serum was added. The plates were

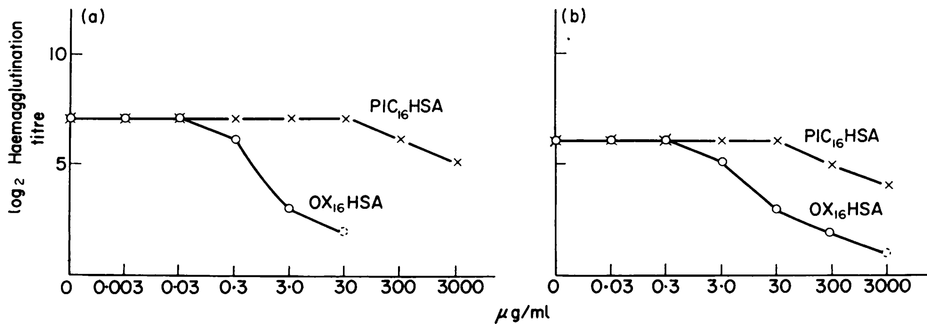

Fig. 2. Specificity of haemagglutinins of lightly and heavily coated Ox-SRBC in (a) oxazoione-immune and (b) nonimmune mouse sera determined by serially diluting in various concentrations of inhibiting $Ox_{16}HSA$ (○) and $Picryl_{16}HSA$ (×). The cells in (a) are those from Fig. 1a and the cells in (b) are those from Fig. 1d.

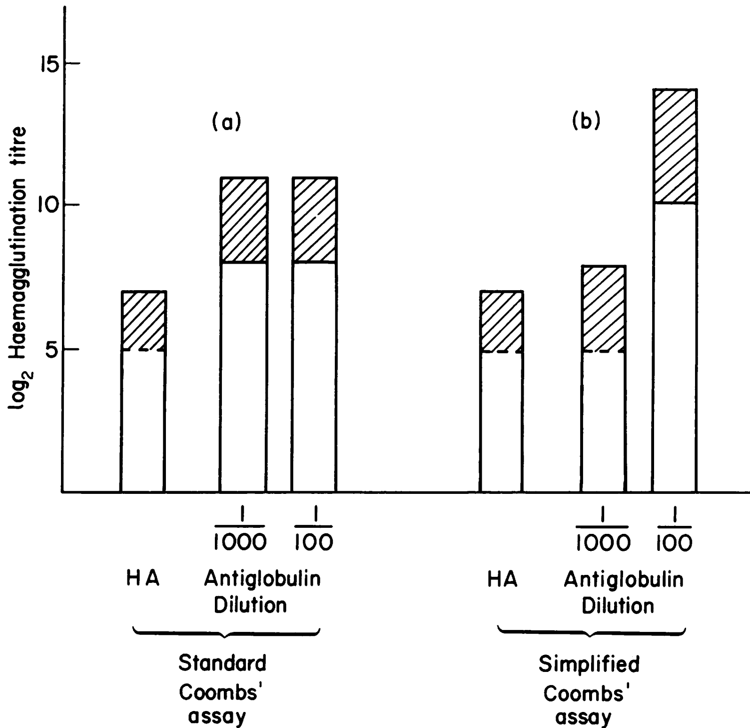

Fig. 3. Direct and antiglobulin augmented haemagglutination titres of (diagonally hatched bars) oxazolone-immune and (open bars) non-immune sera by standard and simplified assays. Standard (a) and simplified (b). Antiglobulin tests employed antimouse globulin. Titres marked HA are unaugmented. Dotted lines indicate that the titre was below the starting solution.

incubated and read as before. With washing eliminated, mouse test serum immunoglobulins that did not react with the coated cells were free to inhibit the antiglobulin. These free inhibitory immunoglobulins were overcome by using high concentrations of antiglobulin. Fig. 3b shows that the simplified antiglobulin assay augments agglutination when concentrated (1:100) antiglobulin is used, but not with dilute antiglobulin (1:1000) while in the standard assay antiglobulin sera at 1:100 and 1:1000 were equally effective (Fig. 3a). When the simplified assay was used to detect oxazolone antibodies in the sera of unimmunized mice, even higher concentrations of antiglobulin reagent were required. This was because nonimmune sera gave no direct haemagglutination with the moderately conjugated cells used in the antiglobulin assay. Therefore, the wells requiring antiglobulin augmentation tended to occur early in the titration where free inhibiting mouse immunoglobulin concentration was especially high, resulting in prozones. Table 1a and b shows

Table 1

Antiglobulin augmented haemagglutination titration patterns of oxazolone immune and non-immune sera

		Test sera dilution: $10^2 \times 2^N$											Anti-light chain dilution
		1	2	3	4	5	6	7	8	9	10	11	
Oxazolone immune sera	a	4^+	4^+	4^+	4^+	4^+	4^+	3^+	2^+	2^+	1^+	0	1:100
	b	4^+	4^+	3^+	3^+	2^+	2^+	1^+	1^+	0	0	0	1:20
Nonimmune sera	c	0	0	1^+	1^+	0	0	0	0	0	0	0	1:100
	d	3^+	3^+	2^+	1^+	0	0	0	0	0	0	0	1:20

Pooled sera from CBA mice 7 days after contact sensitization with oxazolone and pooled sera from unimmunized controls were serially diluted in microtitre wells starting at a concentration of 1:200. Then 0·1 per cent oxazolonated SRBC in a 1:20 or 1:100 dilution of rabbit anti-mouse light chains, were added. 4^+ indicates complete haemagglutination and 1^+ is minimal agglutination.

that there was no prozone when oxazolone immune serum was titrated. However, even with relatively concentrated (1:100) anti-light chain reagents, the titration of nonimmune serum shows two weak positives following two negative wells (Table 1c). This prozone was eliminated when the antiglobulin reagent was used at very high concentration (1:20) (Table 1d). Thus, when the simplified assay was used to test low titred sera from unimmunized mice, higher concentrations of antiglobulin were required to show agglutination than when immune mouse sera were tested.

IMMUNOGLOBULIN CLASS OF ANTI-OXAZOLONE ANTIBODIES IN THE SERA OF IMMUNIZED AND UNIMMUNIZED MICE

Pooled normal and immune sera were serially diluted and Ox-SRBC added in various antiglobulin reagents diluted 1:500, 1:100 and 1:20, show that the titre of immune serum was augmented by antibody to IgM, IgG_1, IgG_{2a} and IgG_{2b}, as well as anti-light chains, using a 1:500 antiglobulin dilution. There was no augmentation by anti-IgA. Under these conditions normal mouse serum gave no reaction. However, there was evidence of antibody in the IgG_1 class of normal sera when 1:100 antiglobulin serum was used with microscopic detection of prozones and 1+ agglutination. Furthermore, under similar conditions 1:20 diluted antiglobulin revealed IgG_{2a} and probably IgM oxazolone

TABLE 2

IMMUNOGLOBULIN CLASS OF ANTI-OXAZOLONE ANTIBODIES OF IMMUNE AND NON-IMMUNE SERA

Augmenting antiglobulin	Titres of oxazolone immune sera (Antiglobulin dilution) 1:500	1:100	1:20	Titres of non-immune sera (Antiglobulin dilution) 1:500	1:100	1:20
Normal rabbit sera*	2†	2	2	<0	<0	<0
Anti-IgA	2	1	2	<0	<0	<0
Anti-IgM	7	5	4	<0	1	1
Anti-IgG_1	8	9	6	<0	4	2
Anti-IgG_{2a}	9	11	6	<0	<0	3
Anti-IgG_{2b}	5	4	4	<0	<0	<0
Anti-light chains	6	8	7	<0	3	3

* Control titres of unaugmented haemagglutination.
† Titres are $1:10^2 \times 2^N$; therefore, '2' is a titre of 1:400.

antibody in the sera of unimmunized mice as well. These higher concentrations of antiglobulin also detected antibody in immune sera that was in all cases three or more tubes higher than in the normal sera.

INHIBITION OF THE SIMPLIFIED ANTI-GLOBULIN TEST BY FREE ANTIGEN

It was possible that the high titres found by the antiglobulin test were due to 'natural' SRBC antibodies which had not been absorbed. This seemed unlikely when it was shown that oxazolone-immune sera absorbed three times with SRBC had no detectable antibody using uncoated SRBC in the antiglobulin test. It was also possible that the high titres were caused by mouse antibodies to sheep erythrocyte antigens revealed by the oxazolonation procedure. To exclude this possibility, unabsorbed normal and oxazolone-immune sera were serially diluted in 0·3 mg/ml Ox-HSA and left for 1 hour before adding the mixture of oxazolonated erythrocytes and the various antiglobulin reagents. All the titres were reduced by more than ten tubes. It was concluded that the simplified antiglobulin augmentation assay was detecting antibody to oxazolone.

DISCUSSION

This report provides methods for the study of mouse antibody to oxazolone and shows that antibody occurs in serum of unimmunized CBA mice. That these normal serum factors are antibodies is based on: augmentation of titres with rabbit antisera specific for mouse light chains and some heavy chains, and inhibition of direct and antiglobulin augmented haemagglutination titres by oxazolonated HSA, to a significantly greater extent than by picrylated HSA (Fig. 2).

Circulating antibodies to other substances containing aromatic rings have also been described in apparently unimmunized animals. These include procaine amide (Russell, 1969), penicillin (Levine *et al.*, 1966), and the dinitrophenyl (DNP) determinant (Brandriss, 1969). The occurrence of mouse (Eisen *et al.*, 1970) and human (Terry *et al.*, 1970) myeloma proteins that combine with DNP groups also suggests that there are a significant

number of lymphoid cells potentially able to make antibodies that can bind aromatic compounds.

Antibody was only detected in normal sera by direct haemagglutination using Ox-SRBC prepared at a high ratio of oxazolone to cells. It is unlikely that this requirement for highly oxazolonated cells was due to a predominance of natural IgM antibody, since it has been shown that IgM antibody requires less antigen density for agglutination (Leikola and Pasanan, 1970). With lightly oxazolonated cells, antibody was only detected in the sera of unimmunized mice by antiglobulin augmentation; this accords with the finding that antigen site density is less critical with antiglobulin augmentation (Hoyer and Trabold, 1970).

There is evidence that low avidity haemagglutinating antibodies are more readily detected by red cells with a high surface concentration of antigen, while the titres of high avidity antibodies are less affected by surface antigen density (Leikola and Pasanan, 1970; Levine and Levytska, 1967). Direct haemagglutination only detected oxazolone antibodies in nonimmune sera when highly coated cells were used (Fig. 1d). This suggests that these antibodies in the sera of unimmunized mice are of low avidity. Conversely, the antibodies in immune sera that agglutinate light oxazolonated cells are probably of higher avidity. Haemagglutinating titres of immune sera are also enhanced when highly coupled cells are used. The finding that these augmented titres are only inhibited with high concentrations of Ox-HSA suggests that the titre was increased through recruitment of low avidity antibodies of immune sera.

Why low avidity antibody needs highly coated cells for agglutination is not clear. It could be that in the restricted area of surface contact between two agglutinating cells the low avidity antibody needs a greater number of antibody molecules linking the cells than high avidity antibody, and there is a greater chance of this with highly coated cells. The antibodies of low avidity found in the sera of unimmunized mice may be the products of cells that are potentially capable of responding to oxazolone or they may be antibodies cross reacting with oxazolone and of high avidity for another ligand to which the mouse is hyperimmune (Little and Eisen, 1969). In the latter case, the more highly coated cells may contain increasing heterogeneity of the antigenic determinant, making interaction with cross reacting antibodies more likely.

Figs 1 and 2 show that a passive direct haemagglutination assay can be designed to meet particular needs. When the detection of low avidity antibody is desired, highly coupled cells should be used; and conversely when high avidity antibody is sought, or when it is desirable not to detect background low avidity antibody, then lightly coupled cells should be used.

Haemagglutination inhibition (HAI) is a widely used method for detecting antigen. Appreciation of the relationship between antibody avidity and cell surface antigen density can result in an HAI assay designed for maximum sensitivity. Fig. 1 viewed as an HAI assay shows that mouse sera 1 week after oxazolone sensitization detects 3·0 and perhaps 0·3 mg/ml Ox-HSA using highly coupled cells (Fig. 1d), while the same sera can detect 0·03 mg/ml Ox-HSA (Fig. 1a) and even 0·003 mg/ml Ox-HSA (Fig. 2a) using lightly coated cells.

Thus, the sensitivity for detection of antigen by HAI, as in radioimmunoassays, should depend on the pre-selection of antisera containing highly avid antibodies and, in addition, on the use of lightly coated cells so that low avidity antibodies needing high concentrations of antigen for inhibition are not involved. When mouse sera hyperimmune to oxazolone

were pre-selected by their ability to agglutinate lightly coated cells, these presumably higher avidity antibodies could detect as little as 30 ng/ml of Ox-HSA (Askenase, unpublished observations).

By eliminating washing of sensitized cells, the antiglobulin test can be performed in one step by micromethods. However, the simplified test should not be used with a non-gamma serum. When the assay was performed with anti-whole mouse sera, mouse albumin adhering to oxazolone coated cells caused very high titred agglutination with both normal and immune sera. This was not seen in the standard test where the cells are washed. Furthermore, the deletion of washing resulted in weakening or inhibiting immunoglobulins, especially with low titred, low avidity antibody of unimmunized mice. This was overcome by using higher concentrations of antiglobulin or by reading the plates under a microscope and using a 'blind' observer or free antigen inhibition to verify minimal agglutinations. The endpoints obtained were reproduceable within a tube when two observers read independently. The simplified assay provides an easy and sensitive method for assessing the relative contribution of each class of immunoglobulin in an antiserum without fractionation, and is suitable for testing large numbers of sera.

The results of this study show that antibodies in all classes, except IgA, can be detected in mice contact-sensitized with oxazolone. Preliminary *in vitro* results from this laboratory indicate that some of these classes of antibodies from contact sensitized mice contain cytophilic antibodies specific for oxazolone (Askenase, 1971). This now draws attention to the problem of which classes of antibody are important in those aspects of contact sensitivity which are due to serum and to macrophages coated with cytophilic antibodies.

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