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Distribution of immunogenic cells after painting with the contact sensitizers fluorescein isothiocyanate and oxazolone. Different sensitizers form immunogenic complexes with different cell populations

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Summary. The distribution of fluorescent cells in the draining lymph nodes of mice painted with the contact sensitizing agent fluorescein isothiocyanate (FITC) was investigated using a fluorescence-activated cell sorter. Up to 30% of the cells were fluorescent after 18 h and this decreased thereafter becoming undetectable after 4–5 days. Most of the fluorescent cells were morphologically lymphocytes, $\theta-$ve and adherent to nylon wool. Immunogenicity of these cells was tested by injecting them into the footpads of normal mice and measuring contact sensitivity after 6 days. This was restricted to large cells which represented less than 5% of the white cell population and nearly all of which became fluorescent after skin painting. The large fluorescent cells were a mixture of monocytes and lymphocytes. Most of the lymphocytes had surface immunoglobulin. The immunogenicity was reduced by nylon filtration but was not affected by silica and anti-θ. These results showed that the immunogenicity is not associated with T cells. In contrast, similar immunogenic activity in the draining lymph nodes of mice painted with oxazolone is associated with T cells. The results therefore showed that different sensitizers form immunogenic complexes with different cell populations, perhaps in this case because of the different water solubilities of FITC and oxazolone. They also suggested that this may cause important differences in antigen presentation, for example in their association with different MHC products.

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INTRODUCTION

Cells taken from the draining lymph node of mice 1–4 days after painting with contact sensitizing agents induce contact sensitivity when injected into normal recipients (McFarlin & Balfour, 1973; Asherson & Mayhew, 1976). Evidence shows the injected cells immunize by transfer of antigen rather than transfer of potential effector cells (Asherson & Mayhew, 1976). This immunogenicity is thought to be important because small numbers of cells can induce the same degree of sensitivity as skin painting whereas conjugates of other self components are not immunogenic (Parker, Aoki & Turk, 1970; Søeberg, Sumerska, Binns & Balfour, 1978) or require adjuvants to induce sensitivity (Landsteiner & Chase, 1941; Eisen, Orris & Belman, 1952; Gell & Benacerraf, 1961; Eisen, Kern, Newton & Helmreic, 1959).

The immunogenic cells in the draining lymph nodes

of mice painted with the sensitizer oxazolone have been shown to be T cells and, to a lesser degree, macrophages but not B cells (Asherson, Zembala & Mayhew, 1977). To study these immunogenic cells further we have developed a model using fluorescein isothiocyanate (FITC) as a sensitizer and a fluorescence-activated cell sorter to monitor and recover fluorescent cells. It was found that fluorescence and immunogenicity was associated with B cells or monocytes and not T cells. This contrasts with the results obtained with oxazolone showing immunogenicity mainly associated with T cells. The different populations of immunogenic cells for oxazolone and FITC may reflect differences in their water solubilities and suggests that contact sensitizers may be divided into groups of T and non-T which may elicit different types of responses.

MATERIALS AND METHODS

Mice

Six- to twelve-week old male CBA, or nude CBA, bred at the Clinical Research Centre were used.

Application of sensitizers

Fluorescein isothiocyanate (FITC) (BDH Chemicals Ltd, Poole, England) (isomer 1) was dissolved to 0·5% (w/v) in a 50/50 (v/v) acetone/di-*n*-butylphthalate (BDH) mixture just before application. Oxazolone (2-phenyl-4-ethoxymethylene oxazolone) (BDH) was dissolved to 3% in ethanol. Both agents were applied to the forepaws and the shaved thorax and abdomen of the mice, oxazolone in 0·1 ml and FITC in 0·4 ml.

Cell preparations

The inguinal, axillary and subscapular lymph nodes were harvested and pressed through wire mesh, washed with medium (Eagle's minimal essential (GIBCO) diluted 50/50 with PBS) and filtered through nylon gauze. For cell sorting, 10% heat-treated foetal calf serum (56° for 30 min) was included in the medium.

Analysis and separations using a fluorescence-activated cell sorter

The light scattering and fluorescence properties of cells was examined by using a fluorescence-activated cell sorter (FACS-1), Becton-Dickinson FACS Systems, Mountain View, Ca, U.S.A.) with the laser set at 488 nm, 200 mW and the photomultiplier at 650 V (Herzenberg & Loken, 1975; Edwards, 1979). For analysis cells were processed at a rate of approximately 1000/s and for cell sorting between 2500 and 3000 cells/s.

Detection of contact sensitivity

Mice were challenged on both sides of both ears with 1% oxazolone in olive oil or 0·5% FITC in acetone/dibutylphthalate and the increase in ear thickness produced by the challenge measured with an engineers' micrometer after 24 h. Results are expressed as units of 10^{-3} cm.

Assay for the immunogenicity of lymph node cells

Cells from lymph nodes of mice painted one day previously with FITC or oxazolone were injected into each hind footpad of normal mice. Usually 10^6 or 2×10^6 cells were injected in a volume of 0·1 ml/mouse. After 6 days the mice were challenged on the ear with antigen and assessed for contact sensitivity 24 h later. Previous studies (Asherson & Mayhew, 1976) have shown that the induction of sensitivity is not inhibited by treating the injected cells with metabolic inhibitors, for example emetine, which irreversibly inhibits protein synthesis.

Treatment of cells with anti-θ serum

The preparation and use of anti-θ (AKR anti-C3H) and complement has been described in detail (Asherson, Allwood & Mayhew, 1973). Treatment with anti-θ and guinea-pig complement killed about 70% of cells from lymph nodes.

Nylon wool filtration

Cells were passaged through nylon wool columns according to Cantor & Simpson (1975). Control preparations were incubated and centrifuged in parallel. Cells recovered after passage were about 90% sensitive to anti-θ and complement in the experiments reported.

Incubation of cells with silica

Cells were incubated at 10^7/ml in medium with 10% foetal calf serum, and 10 μg/ml silica-powdered quartz (Dorentrop; lot DG12, particle size less than 5 μm) for 6 h (Asherson *et al.*, 1977).

Fluorescent antibody

Cells at about 10^8 per ml were incubated with a 1/10 dilution of rabbit anti-μ for 30 min in an ice bath. The cells were washed twice and then incubated with an appropriate dilution of FITC-goat anti-rabbit Ig (Behring Institute, Frankfurt, West Germany).

Irradiation

Cells were irradiated *in vitro* from a ^{60}Co source at about 1 rad/s.

RESULTS

Detection of fluorescent cells in draining lymph nodes after painting the skin with fluorescein isothiocyanate (FITC)

Mice were painted with FITC and after 18 h cells from draining lymph nodes were washed and examined with an FACS. Two fractions of cells based on light scatter (size) (Fig. 1) were studied, the lymphocyte fraction

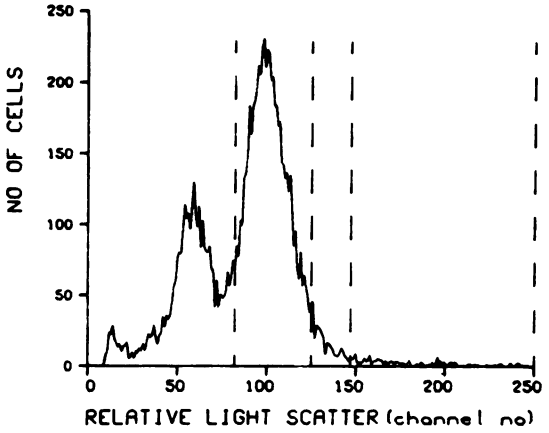

Figure 1. Light scatter distribution of cells from draining lymph nodes. The two peaks on the left contained platelets and red cells respectively. Morphologically cells in the next fraction were predominantly lymphocytes (channels 82–125) and the large cell fraction (channels 147–250) contained lymphocytes and monocytes. Scatter gain = 8.

which forms the largest peak of the light scatter distribution and a fraction of larger cells. Stained preparations showed nearly all cells in the lymphocyte fraction were lymphocytes and the large cell fraction consisted of a mixture of lymphocytes and monocytes. Cells in the smaller fractions which include red cells and platelets did not show fluorescence and have not been examined further. Cells in the lymphocyte fraction (60–70% of the total cells) and cells in the large cell fraction (1–3%) showed fluorescence after painting with FITC and the number of fluorescent cells and the intensity of fluorescence could be increased by painting a larger dose (Fig. 2). For comparisons a reference point of fluorescence was chosen such that about 99% of cells from normal mice were negative. From this reference point 30% of the lymphocyte fraction became fluorescent after painting with 4 mg of FITC (Table 1). At least 60% of the larger cells became positive, this analysis being complicated because large cells had a higher background than the lymphocyte fraction.

A time course for the presence of fluorescent cells was studied by examining cells from the draining lymph nodes and spleen at daily intervals after painting with 2 mg FITC (Fig. 3). The spleen had fewer positive cells than the draining lymph nodes. By 4–5 days after painting the frequency of fluorescent cells, both in the lymphocyte and large cell fractions, was indistinguishable from that of normal mice.

Characterization of fluorescent cells

The effect of nylon wool filtration, and anti-θ treatment on the frequency of fluorescent cells in the lymphocyte fraction was studied. Table 2 shows that anti-θ and complement increased the percentage of positive cells whereas nylon wool removed the positive cells (or their fluorescence). The effect of these treatments on cells taken 1 or 3 days after painting was the same. Fluorescent cells collected from the lymphocyte fraction were stained with May Grünwald Giemsa and had the morphology of lymphocytes. The properties of the large cell population, which were only a small percentage of the white cells were examined by different techniques and will be described in the next section.

Properties of immunogenic cells

Cells from the draining lymph nodes injected into the footpads of mice induced sensitivity (ear swelling reactions) similar to that generated by skin painting (Table 3). Fluorescent cells collected from the lymphocyte fraction from painted mice were, however, consistently unable to induce sensitivity. Therefore cells were separated into the lymphocyte and large cell fractions and injected according to their proportion in the starting population. Mice injected with cells from the large cell population (4% of the white cells in the experiment shown) developed the same degree of sensitivity as unfractionated cells (Table 4) while cells from the lymphocyte fraction were inactive.

The immunogenicity was resistant to silica treatment (5 h 37°, 100 μg/ml), irradiation (2000 rad) and anti-θ treatment but was removed by nylon wool filtration (Table 5). These results differed from those

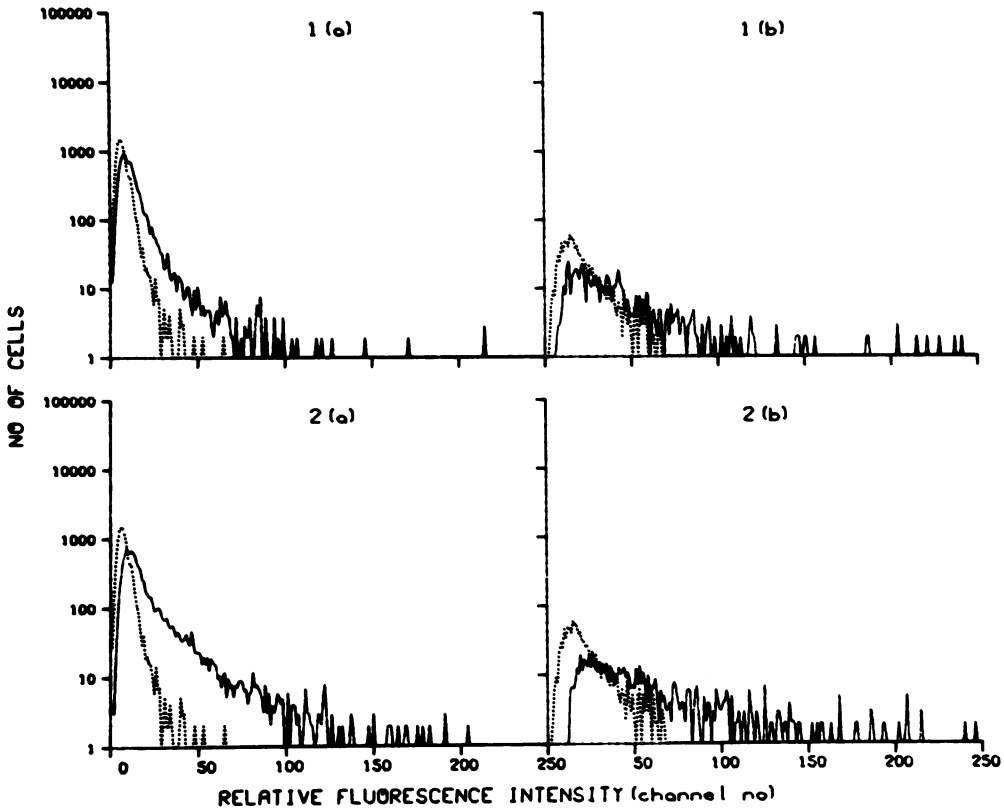

Figure 2. Fluorescence of cells in draining lymph node after painting with FITC. Fluorescence intensity of the lymphocyte fraction from mice painted 18 h previously with 2 mg FITC (1a) and 4 mg FITC (1b). Fluorescence of large cells after 2 mg and 4 mg of FITC are shown in (2a) and (2b). (- - -) cells from untreated mice; (——) cells from painted mice. 10,000 cells in the lymphocyte fraction were counted but because of the low numbers only 2000 large cells were counted. Fluorescence gain = 16.

Table 1. Fluorescent cells in lymphocyte and large cell fractions 18 h after painting with FITC

	% Fluorescent cells	
FITC (mg)	Lymphocyte fraction	Large cells
0	1·9	44·9
2	14·1	85·2
4	29·9	94·7

Results show the percentage cells which were fluorescent after correction for background fluorescence as defined by lymphocytes from unpainted mice. 10,000 cells were counted in the lymphocyte fraction and 2000 cells in the large cell fraction.

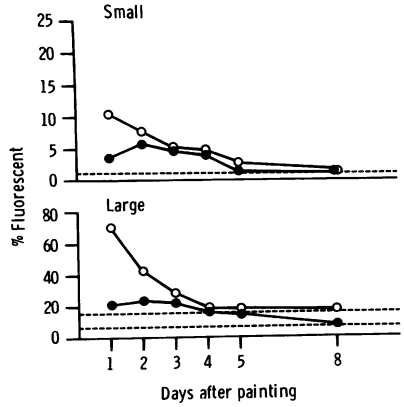

Figure 3. Decay of the presence of fluorescent cells in draining lymph nodes (○) and spleen (●) after painting with FITC. The frequency of fluorescent cells with respect to a standard reference point is shown (% fluorescence) at various days after painting with 2 mg FITC. The lymphocyte fraction (small) and large cell fraction (large) have been described (Fig. 1, Table 1). Results show the mean of two mice. Two backgrounds (- - -) for large cells are shown because this value was less for spleen cells.

Table 2. Characteristics of fluorescent cells in lymphocyte fraction

	% Fluorescent cells	
	day 1	day 3
Normal (no paint)	1·1	
FITC untreated	6·9	1·7
Nylon filtered	2·5	0·4
Incubated, 20% FCS (nylon control)	6·3	2·0
Anti-θ + C′	16·1	3·1
C′	3·4	1·1

Cells taken from lymph nodes 1 and 3 days after painting with FITC were treated as indicated and 10,000 cells in the lymphocyte fraction counted and scored as fluorescent with respect to a reference point showing 1·1% of normal cells as positive.

Table 3. Ability of cells from the draining lymph nodes of mice painted with FITC to induce contact sensitivity

	Contact sensitivity (ear swelling, 10^{-3} cm)
Normal mice	1·4 (1·4)
Painted 2 mg FITC	5·8 (1·7)
2×10^6 cells*	5·8 (1·9)

* Cells were taken from draining lymph nodes 1 day after painting with 2 mg FITC and injected into hind footpads (2×10^6 cells/mouse). The mice were challenged on the ears with FITC 6 days after injecting cells, or 7 days after painting, and their swelling reaction measured. Results show mean (SD) of groups of five.

Table 4. Contact sensitivity induced by large cell and lymphocyte fraction

	Ear swelling (10^{-3} cm)
Untreated cells (10^6/mouse)	5·3 (0·5)
'Lymphocyte' fraction ($0{\cdot}96 \times 10^6$/mouse)	0·9 (0·5)
Large cells ($0{\cdot}4 \times 10^6$/mouse)	5·5 (1·0)
No cells	1·6 (0·7)

Cells were taken from draining lymph nodes 1 day after painting with FITC and sorted into large cell and lymphocyte fractions. They were injected into recipients according to their proportion in the population. Results are mean (SD) of groups of four to five.

Table 5. Characteristics of immunogenic cells

Treatment	Ear swelling (10^{-3} cm)
(a) Incubation (20% FCS) (nylon control)	7·6 (1·8)
Nylon filtration	2·3 (1·2)
Incubation (20% FCS) (silica control)	7·7 (1·8)
Silica	6·6 (2·4)
2000 rad	6·0 (3·4)
No cells (−ve control)	0·7 (0·3)
(b) Complement	6·2 (1·3)
Anti-θ and complement	5·3 (0·5)
Nylon	2·4 (1·5)
Incubation (20% FCS) (nylon control)	4·5 (1·9)
No cells (−ve control)	1·5 (0·5)

Cells were taken from the draining lymph nodes 18 h after painting with FITC and treated as indicated. They were then injected into groups of four to five normal mice (10^6/mouse) and their ability to induce sensitivity as measured by ear swelling tested. Results from two experiments (a and b) are shown.

Table 6. Effect of anti-θ and silica on the ability of cells from FITC or oxazolone painted mice to induce sensitivity

	Ear swelling (10^{-3} cm)
(a) FITC	
No cells (−ve control)	0·7 (0·4)
Incubated 20% FCS (silica control)	2·1 (0·5)
Silica	1·9 (0·9)
Complement and silica	1·9 (0·8)
Anti-θ + complement + silica	3·4 (1·2)
Anti-θ + complement	4·0 (0·5)
Complement	2·0 (0·4)
(b) Oxazolone	
No cells (−ve control)	6·6 (1·6)
Complement	12·5 (2·1)
Anti-θ + complement	5·6 (0·7)

Cells (10^6/mouse) from draining lymph nodes of mice painted with FITC or oxazolone were treated as shown and injected into groups of four to five normal mice and their ability to sensitize measured after challenge with either FITC or oxazolone.

previously obtained with oxazolone. Experiments with cells from mice painted with FITC or oxazolone were therefore performed in parallel (Table 6). Anti-θ abolished the ability of cells from oxazolone painted mice to induce sensitivity but anti-θ or anti-θ + silica had no effect on the sensitivity induced by cells from FITC painted mice. The lack of requirement for T cells

Table 7. Contact sensitivity induced by the injection of cells from nude mice painted with FITC

	Ear swelling 10^{-3} cm
No cells	0·3 (0·3)
CBA	2·9 (0·8)
CBA nude	3·7 (1·0)

Results are mean (SD) of ear swellings of groups of five mice injected with 2×10^6 cells.

was also shown by the immunogenicity of cells from CBA nudes (Table 7).

The large fluorescent cells were collected with the FACS, then stained with May Grünwald Giemsa and for surface Ig by immunofluorescence. Immunofluorescence was possible with fluorescein conjugate antiserum because cells from FITC painted mice were not fluorescent by UV microscopy. By counting 200 cells it was found that 45% of the cells resembled lymphocytes and the rest monocytes. Thirty-six per cent of cells from the same preparation showed surface Ig.

DISCUSSION

Cells taken from the draining lymph nodes of mice painted with contact sensitizers and injected into the footpads of normal recipients induce contact sensitivity equivalent to that induced by the painting. Several lines of evidence show that this is an immunizing process caused by an antigen–cell complex (Asherson & Mayhew, 1976): (1) the sensitivity is best produced with cells taken 1 day after painting and declines thereafter; (2) the recipients require several days to become sensitive; (3) the activity of the cells is not affected by irreversible inhibitors of macromolecular synthesis including protein synthesis. Viable cells are however required as well as some MHC compatibility between donor and recipient (Asherson, Mayhew & Perera, 1979). The cells inducing contact sensitivity in a study using oxazolone were shown to be T cells, and to a lesser extent phagocytic cells but not B cells (Asherson, *et al.*, 1977).

To investigate this inductive phase further we used FITC as a sensitizer so that the presence and persistence of cell–antigen complexes could be followed with the FACS. Based on light scattering, two populations of cells were considered, the lymphocyte fraction which composed 96–99% of the white cells and a fraction of larger cells. Both fractions became fluorescent after skin painting and lost the label after 4–5 days. Up to 30% of the lymphocytes could be labelled. They were B cells in that they were morphologically lymphocytes, resisted anti-θ treatment and adhered to nylon wool. These cells which formed about 90% of labelled white cells were not immunogenic when injected into the footpads of normal mice. It should be noted that cells need to bind about 5000 molecules of FITC for detectable fluorescence (Loken & Herzenberg, 1975).

The large cells, which nearly all became fluorescent after skin painting, were immunogenic and produced contact sensitivity equivalent to that produced by skin painting when injected in a dose proportional to their occurrence in draining lymph nodes. The immunogenicity was resistant to anti-θ and silica treatment (even when combined) but was removed by passage through nylon wool. Nude mice could also be used for donors of immunogenic cells. These results were unexpected because previous studies with oxazolone showed immunogenic cells to be T cells and to a lesser extent phagocytic cells (Asherson *et al.*, 1977). The previous studies with oxazolone were repeated and confirmed that almost all of the immunogenicity of cells from mice painted with oxazolone was removed by anti-θ serum and complement. Further tests showed the large fluorescent cells were a mixture of lymphocytes positive for surface Ig and monocytes.

It therefore appears that FITC associated with B cells or monocytes in the draining lymph nodes and that these cells are immunogenic. The T cells presumably are not immunogenic for FITC, as they are for oxazolone, because they do not bind or retain sufficient quantities of antigen. This is not an inherent inability of T cells to bind FITC because they can become fluorescent after intravenous injection of FITC (Thomas, Edwards, Watkins & Asherson, unpublished results). The difference between oxazolone and FITC may be because oxazolone is lipophilic and can dissolve in the lipid of the skin and lymph and then deposit in the T-cell areas of the lymph nodes, as described for other lipophilic compounds (Coon & Hunter, 1973). FITC is water soluble which may influence its distribution. It is also possible that the preferential trapping of oxazolone in T-dependent areas explains why it is able to induce more sensitivity (sensitize for larger ear swellings) than FITC.

The immunogenic cells in the lymph nodes and lymph appear to be the important inducers of contact sensitivity. Lymph node drainage and lymph nodes

(Frey & Wenk, 1957; Turk & Stone, 1963; Asherson, Zembala & Mayhew, 1974) are required for the induction and both free and cell-bound sensitizer can be detected in the lymph and lymph nodes (Hall & Smith, 1971; Nakagawa & Amos, 1977; Nakagawa, Amos, Gotoh & Tanioku, 1969; Parker & Turk, 1970). Conjugates of sensitizer and skin or serum proteins are poor immunogens (McFarlin & Balfour, 1973; Parker *et al.*, 1970; Søeberg *et al.*, 1978) and require adjuvants for any immunogenicity (Landsteiner & Chase, 1941; Eisen *et al.*, 1952, 1959; Gell & Benacerraf, 1961), in contrast to the strong immunogenicity of cell–antigen complexes (McFarlin & Balfour, 1973; Søeberg *et al.*, 1978) which induces the same amount of contact sensitivity as skin painting (Thomas, Asherson & Perera, 1978). The finding that sensitizers with different physico-chemical properties form immunogenic complexes with different cell populations has important implications. As already suggested the ability of lipid soluble sensitizers to form immunogenic complexes with T cells may promote the sensitizing ability. It could also be expected that the sensitizers on different cell poplations would be presented in association with different surface antigens including Ia. The distinction of whether a sensitizer binds to T cells or non-T cells may therefore affect the regulation of these responses.

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