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Elution of Macrophage-Bound Immunoglobulins by Temperature Changes *in vitro*

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Summary. Some mouse anti-sheep-erythrocyte macrophage cytophilic antibodies are more readily adsorbed to cells at 4° than at 37°. By treating cells with serum at 4°, washing them well and eluting bound material at 30°, it was found possible to isolate cytophilic immunoglobulins. These were found to have fast γ-mobility, but were heterogeneous upon preparative ultracentrifugation and gel-filtration. The macrophage eluate contained relatively high titres of anti-sheep-erythrocyte cytophilic antibodies.

INTRODUCTION

In spite of having been first described over 10 years ago (Boyden and Sorkin, 1960), macrophage cytophilic antibodies still present many problems to the investigator attempting to define their role in the living animal. Not the least of these problems is the observation, (Boyden, 1963; Tizard, 1969; Nelson, 1969) that in some species, notably the mouse, macrophage cytophilic antibodies may be adsorbed more readily by macrophages, *in vitro* at 4° than at 37°. In the experiments reported here, use was made of this property to isolate and characterize macrophage-bound immunoglobulins in mice.

MATERIALS AND METHODS

Mouse anti-sheep erythrocyte serum

Serum containing macrophage cytophilic antibodies was raised in White Swiss mice by two intraperitoneal injections of 0·1 ml, 10 per cent sheep erythrocytes in saline, 1 month apart. Mice were bled 6 days after receiving the second dose of antigen.

Titration of sera for the presence of cytophilic antibodies, opsonic adherence activity, haemagglutinating activity and haemolytic activity was performed as described previously (Tizard, 1969).

Mouse peritoneal cells

When used for elution experiments, peritoneal cells were flushed from the peritoneal cavities of normal mice with 4 ml, sterile normal saline containing 20 i.u. heparin (Connaught Labs.). When used for cytophilic antibody or opsonic adherence titration the peritoneal cavities were washed out with 4 ml Hanks's BSS containing 0·5 per cent bovine serum albumin (Nutritional biochemicals).

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Elution of cytophilic material from peritoneal cells

One ml of mouse serum containing anti-sheep-erythrocyte cytophilic antibodies was mixed with the pooled, packed, peritoneal cells from thirty normal mice. This suspension was incubated for 90 minutes at 4°. At the end of this time the suspension was filtered through a 1 cm × 1 cm bed of Sephadex G-25 medium (Pharmacia) so that the cells alone were retained. The temperature of this gel-bed and the phosphate-buffered saline used to wash it were maintained below 5° by an ice jacket. The gel-bed containing the cells was then washed through with cold PBS (25 ml/hour) until no more protein was detectable in the effluent. (As measured by absorption at 254 mμ.) The flow of cold PBS was then stopped, the ice in the jacket replaced by water and the temperature of the gel-bed raised to 30°. After allowing the system to stabilize at this temperature for 10 minutes the flow of warm PBS was recommenced. The effluent was monitored until no further material, absorbing at 254 mμ, was released.

RESULTS

Elution of protein from peritoneal cells

It was found that the rise in temperature of serum-treated cells from 4° to 30° resulted in elution of material which could be detected by its absorption at 254 mμ. When its

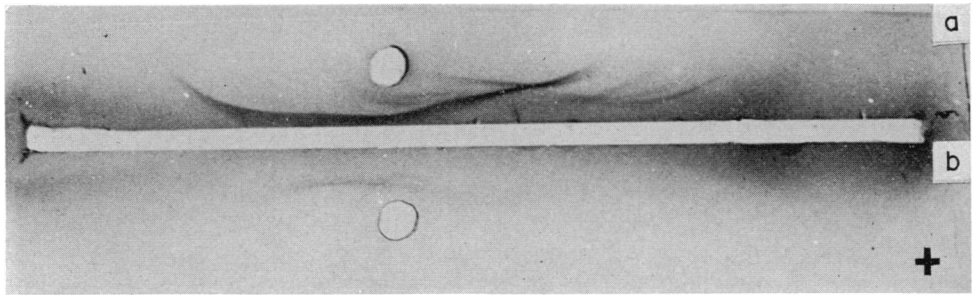

FIG. 1. Immunoelectrophoresis of (a) mouse serum globulins and (b) eluate from serum-treated mouse peritoneal cells, against rabbit anti-mouse serum.

absorption spectrum was plotted it was found to absorb maximally at 278 mμ. The amount of this material, as protein, released after the exposure of 8×10^7 peritoneal cells to 1 ml serum in ten experiments varied between 15 and 34 μg per 10^6 cells (mean 23 μg). Peritoneal cells which had not been preincubated in serum at 4° *in vitro* also released some protein (5 μg per 10^6 cells) after being cooled to 4°, washed and then warmed to 30°.

Physicochemical properties of the eluates

Immunoelectrophoresis of eluates against rabbit-anti-mouse serum indicated that the major component, and in most cases, the only detectable component of the eluate was a globulin of fast γ-mobility. Two of ten eluates tested also contained traces of a second protein which gave a short precipitation line close to the origin. (Fig. 1).

Eluates were examined by preparative ultracentrifugation in a Beckman model L-2 ultracentrifuge for 18 hours at 100,000 *g* on a 5–40 per cent sucrose gradient. This indicated that while most of the eluate was composed of 7S molecules, a fraction was

extremely dense and possibly represented aggregation of the eluted material. A similar, finding occurred when eluates were subjected to gel filtration on Sephadex G-200. Most of the material had a MW in the region of 150,000–200,000 but some was completely excluded from the gel. Antibody activity was confined to the major peak.

TABLE 1

ANTIBODY ACTIVITY IN FOUR ELUATES FROM SERUM TREATED MOUSE PERITONEAL CELLS

Material	Haemagglutination	Antibody activity		
		Opsonic adherence	Cytophilic antibody	Haemolysin
Whole serum	10^6*	10^3	10^3	10^6
Eluate No. 1. 300 μg/ml	2	32	16	8
2. 400 μg/ml	2	16	16	8
3. 390 μg/ml	4	16	16	8
4. 800 μg/ml	2	32	32	32

* Figures represent the highest dilution at which haemagglutination or rosette formation could be detected, or at which 50 per cent haemolysis occurred.

Antibody activity of eluates

Four eluates were tested for haemagglutination, opsonic adherence, cytophilic antibody and haemolytic activity against sheep erythrocytes. Relative to haemagglutinating and haemolytic activity, titres of cytophilic antibodies and opsonic adherence antibodies were raised as compared with whole serum (Table 1). However cytophilic antibody titres were not raised relative to those of opsonic adherence. This may be related to the fact that antigen may be opsonized by cytophilic antibodies whereas opsonic adherence antibodies need not be cytophilic. Thus the opsonic adherence test also measures cytophilic antibodies

TABLE 2

THE ADSORPTION OF ELUATE BY PERITONEAL MACROPHAGES

Temperature (°C)	Protein concentration (μg/ml)	Number of macrophages	Per cent eluate adsorbed
4	400–2·0	$1{\cdot}44 \times 10^6$	$18{\cdot}36 \pm 2{\cdot}12$ (S.D.)
37	400–2·0	$2{\cdot}41 \times 10^6$	$2{\cdot}79 \pm 1{\cdot}75$ (S.D.)

while the reverse is not the case (Tizard, 1970). The presence of haemolytic activity in the eluate may indicate that antibodies other than cytophilic ones were present in the eluate since complement fixation and cytophilic properties are not considered to exist on the same molecule (Nelson and Boyden 1967).

Cytophilic antibody activity in both eluate and whole serum was unaffected by treatment with 0·1 M 2-mercaptoethanol.

Cytophilic activity of eluates

The cytophilic activity of these eluates was further investigated by measuring their uptake by relatively pure suspensions of mouse peritoneal macrophages. Mouse peritoneal

cells were cultured in plastic petri dishes for 48 hours with removal of non-adherent cells at 12-hourly intervals. Four hundred micrograms of eluate protein was labelled with 50 μc ^{131}I by the method of Greenwood, Hunter and Glover (1963) and dilutions of this labelled preparation exposed to suspensions of peritoneal macrophages for 120 minutes; either at 4° or at 37° in the presence of 2×10^{-3} M sodium fluoride to inhibit membrane activity (Weiser, Heise, McIvor, Han and Granger, 1970). At the end of this time, the cell suspensions were centrifuged and the activity in the supernatant compared with the activity in the cell button. Activity was counted in a Packard auto-gamma well type spectrometer.

Over a range of protein concentrations between 400 and 2 μg/ml, approximately 1×10^6 macrophages adsorbed about 20 per cent of the available protein at 4° and about, 1·5 per cent at 37° (Table 2).

DISCUSSION

These results confirm earlier observations (Tizard, 1969; Nelson, 1969) that some mouse anti-sheep-erythrocyte cytophilic antibodies may be adsorbed much more readily to macrophages at 4° than at 37°. Cytophilic antibodies detectable in serum represent only a part of the total cytophilic antibody content of an animal, since presumably a proportion of this antibody is cell bound. It is also probable that these serum cytophilic antibodies represent the least avidly cytophilic proportion of this population, and are not necessarily representative of the population as a whole.

Both Weiser *et al.* (1970) and Zembala and Asherson (1970) have demonstrated that under suitable circumstances, unreactive mouse macrophages may acquire immunological reactivity by exposure to immune serum at 37°.

Additional evidence for heterogeneity among mouse macrophage cytophilic antibodies is the finding that macrophage eluates contained, predominantly, fast migrating γ-globulin. There was no detectable slow migrating γ-globulin in any of the ten eluates tested. Previously mouse IgG cytophilic antibodies have been considered to γ_2 on the basis of the experiments of Parish (1965) who demonstrated that cytophilic antibody activity against bovine plasma albumin and bovine γ-globulin migrated in the slow γ-region on agar block electrophoresis. These differences may be due to several factors.

It is possible that the fast migrating γ-globulin in the cell eluates is not cytophilic antibody, but material derived from the small lymphocytes present in the peritoneal cell population. This is improbable since small lymphocytes do not adsorb cytophilic antibodies and in the absence of serum pretreatment, the peritoneal cells only released a small amount of protein which could not be shown to contain γ-globulin.

Alternatively, the γ-globulin in the eluate may represent a different population of cytophilic antibodies to that measured by Parish, however it has been shown that rosette formation around mouse peritoneal cells mediated by cytophilic antibodies is almost completely prevented at 37° (Tizard, 1970) indicating that if γ_2-cytophilic antibodies do occur, they also are inhibited from cell binding at 37°.

It is most likely that the difference between these results and those of Parish may be related to the use of Freund's complete adjuvant. This has been employed by many workers (Parish, 1965; Nelson and Mildenhall, 1967; Berken and Benacerraf, 1966) to raise cytophilic antibodies in mice, and is known to induce IgG_2 antibody formation rather than IgG_1 (Askonas, White and Wilkinson, 1965).

A comparison of the properties of cytophilic antibodies induced with, and without, adjuvant may reveal other significant differences.

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