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Phagocytosis of Murine Lymphoma Cells by Macrophages

II. DIFFERENCES BETWEEN OPSONIC AND CYTOTOXIC ACTIVITY OF MICE IMMUNIZED WITH LYMPHOMA CELLS

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Summary. Primary allogeneic antiserum raised in C57Bl mice directed against a DBA/2 lymphoma, L5178Y, was found to contain at least two types of opsonic activity associated with the γ-globulin fraction of the antiserum. One was found at a low level corresponding to cytotoxic activity, binding strongly to L5178Y cells and resistant to treatment with mercaptoethanol. The other, which accounted for most of the opsonic activity, was relatively non-specific for L5178Y cells, was easily eluted from the cells, was not associated with cytotoxicity and was sensitive to mercaptoethanol treatment. Two types of opsonic activity were also found in allogeneic antiserum against the CBA/2 lymphoma, TLX5. Both types of lymphoma antisera opsonized sheep red cells, but anti-sheep red cell serum was highly specific, exhibiting no degree of opsonic cross-reactivity with either type of lymphoma cell.

INTRODUCTION

The existence of serum factors which promote phagocytosis of inert as well as antigenic materials is well established. Generally, specific immune serum has a higher concentration of such factors, or opsonins, than serum from normal unimmunized animals. Opsonins appearing as a result of bacterial infection have been studied far more extensively than tumour opsonins; for example, Laxdal, Messner, Williams and Quie (1968) showed that opsonic activity was associated with the 7S-γ-globulin in the serum of patients with subacute bacterial endocarditis, and suggested that this opsonic activity was a more sensitive index of antibody response than agglutinating or complement-fixing activities. In the tumour system, Bennett, Old and Boyse (1963, 1964), using a variety of allogeneic tumour cells, found that cultures of peritoneal macrophages ingested tumour cells in the presence of specific allogeneic antibody. However, apart from the reported high degree of specificity for the immunizing antigen, there is still little information on the precise nature of these tumour opsonins. In the present system, antiserum directed against the DBA/2 lymphoma, L5178Y (Fischer, 1958) was produced in allogeneic C57Bl mice. Experiments were carried out on this serum to establish whether or not opsonic and cytotoxic activity found during the primary immune response were the same or different factors.

MATERIALS AND METHODS

Mice

Ten-week-old C57Bl female mice were used for the production of anti-L5178Y serum, and DBA/2 mice to grow the lymphoma after intraperitoneal injection.

Cells

(i) Lymphoma L5178Y cells grow as a lethal ascites in DBA/2 mice. Cells for all experiments were obtained by washing out the peritoneal contents of DBA/2 mice after i.p. injections of 5×10^5 L5178Y cells 7 days previously.

(ii) Lymphoma TLX5 cells, an ascitic tumour syngeneic in CBA mice, were used during specificity-absorption tests on anti-lymphoma L5178Y serum (see below).

(iii) Fresh sheep red blood cells, washed $\times 3$ in Fischer's medium, were also used in specificity-absorption tests on anti-L5178Y serum.

(iv) Peritoneal macrophages. Their harvesting from C57Bl mice injected 6 days previously with 1 ml of thioglycollate together with the preparation of macrophage slip cultures is fully described in the accompanying paper (Evans, 1971).

Experimental procedures

(a) *Immunization of C57Bl mice.* For the primary immunization, 4×10^6 L5178Y cells were injected i.p. into batches of fifty mice. Serum was collected at different intervals by bleeding from the retro-orbital sinus and pooling the blood. Normal serum was collected at the same time and both immune and normal sera were stored in 1 ml volumes at $-20°$. Secondary immunization involved injecting 10^7 L5178Y i.p. 36 days after the primary injection. All sera were heated at 56° for 1 hour and dialysed overnight against Fischer's medium, unless otherwise stated.

(b) *Cytotoxic test.* The conventional trypan blue exclusion method as a measure of cell viability was used. Two-fold, three-fold or five-fold dilutions of serum were prepared in Fischer's medium in plastic haemagglutination trays. To each dilution, 10^5 lymphoma L5178Y cells were added, followed by one drop of guinea-pig serum, previously absorbed with 10^8 L5178Y cells at 4° and diluted 1/3 with Fischer's medium. The final total volume of the reaction mixture was approximately 0·15 ml. The trays were incubated for 45 minutes, then one drop of 0·1 per cent trypan blue solution was added to one series of dilutions at a time. One drop of each solution with the cells in suspension was placed on a slide and the percentage of cells stained was calculated. The end point was taken as that dilution showing 50 per cent live cells. Controls never showed more than 5 per cent dead cells.

(c) *Opsonic test.* The technique used was a modification of that reported by Bennett *et al.* (1963) and is described in detail elsewhere (Evans 1971). Briefly, serial five-fold dilutions (0·4 ml volumes) of anti-L5178Y serum or normal serum were mixed with 10^5 lymphoma cells, and 0·2 ml volumes of each dilution were added to macrophage slip cultures. These were incubated for 1·5–2 hours and then examined microscopically to determine the degree of phagocytosis. The end point denoting positive phagocytosis was taken as that dilution at which at least 1 per cent of the macrophages had ingested lymphoma cells. Control, normal serum, showed very little or usually no opsonic activity.

(d) *Treatment of serum with saturated ammonium sulphate.* 1 ml volumes of heat inactivated normal and anti-L5178Y serum were mixed with an equal volume of saturated ammonium sulphate. The precipitate was allowed to form at room temperature for 30 minutes and was then deposited by centrifugation. The sediment was washed $\times 2$ with 50 per cent ammonium sulphate followed by redissolving in 1 ml of Fischer's medium. Precipitation, washing and redissolving procedures were repeated twice before the final 1 ml volume was dialysed overnight against Fischer's medium.

(e) *Zone electrophoretic fractionation of serum.* 10 ml of serum collected 14 days after injection of L5178Y cells was dialysed against 0·05 M barbiturate buffer, pH 8·6, and centrifuged. The sample was then applied to a slit cut in the centre line of a Pevikon block, which was then electrophoresed for 36 hours at 20–30 mA current. After this time, the block was cut into 1 cm strips and the various fractions were eluted with buffer. Fractions containing γ-globulins were pooled and kept separate from the non-γ-globulin-containing fractions. Both γ-globulin and non-γ-globulin pools were dialysed after concentration by ultracentrifugation.

(f) *Treatment of serum with mercaptoethanol.* The method described by Hall, Smith, Edwards and Shooter (1969) was used. 2-Mercaptoethanol, 0·2 M (Koch Light Laboratories Ltd, Colnbrook, Bucks.), was made up in 0·15 M phosphate buffer, pH 7·4. Serum samples were mixed with an equal volume of mercaptoethanol solution and incubated at 37° for 15 minutes, after which they stood at room temperature for 4 hours. They were then dialysed against cold, running tap water for 4 hours followed by overnight dialysis against phosphate buffered saline.

(g) *Differences in binding capacity of cytotoxic and opsonic activities.* 1 ml volumes of normal and immune serum, collected 14 days after lymphoma injection, were mixed with 10^8 packed L5178Y cells and incubated for 2 hours at 37°. The tubes were shaken every 15 minutes to resuspend the lymphoma cells. After the incubation period the cells were sedimented by centrifugation and the supernatants were removed. The sedimented cells were next washed twice in 2 ml and finally in 1 ml of Fischer's medium. These washings (5 ml) were pooled. The cytotoxic and opsonic activities of the absorbed serum and washings were assayed and compared with unabsorbed serum suitably diluted.

(h) *Determination of opsonic activity of cytotoxic antibody.* A duplicate series of five-fold dilutions of 14-day immune serum was prepared and 2×10^5 L5178Y cells in 0·1 ml Fischer's medium were added to each tube. The dilutions were then incubated at 37° for 1 hour. Following this, one of the duplicate series of dilutions was centrifuged, the cells washed $\times 3$ in Fischer's medium and finally resuspended in 0·5 ml of medium. The other series of dilutions was not washed. 0·2 ml volumes of each dilution in both series were next added to macrophage slip cultures for the opsonic test, and incubated for 1·5 hours. The remaining 0·1 ml of cell suspension in each tube was mixed with one drop of guinea-pig serum and incubated for 15 minutes. This was to compare the end point dilution of cytotoxic activity with that showing positive opsonic activity.

(i) *Specificity of cytotoxic and opsonic activities.* Primary response antisera against TLX5 and sheep red blood cells were prepared by injecting C57Bl mice i.p. with 4×10^6 TLX5 cells or 1 ml of a 1 per cent suspension of sheep red cells, and collecting the serum 2 weeks later. These sera were shown to be opsonic and cytotoxic towards the specific immunizing antigen. To test the specificity of opsonic and cytotoxic activities and also cross-reactivity towards foreign antigens, five-fold serial dilutions of anti-L5178Y, TLX5 and sheep red blood cell-sera were prepared, and mixed with each of the two foreign antigens. The three reciprocated crosses were made in duplicate. Normal serum was also diluted and mixed with each of the cell types. Each tube contained 0·5 ml of Fischer's medium with 2×10^5 L5178Y, TLX5 cells or 0·1 per cent suspension of sheep red cells. These were then incubated for 1 hour. Following this, the procedure was exactly the same as described in (h), one dilution series of each duplicate being thoroughly washed before being used in the opsonic test, the other not being washed. Guinea-pig serum, as a source of comple-

ment, was added to the cells remaining in each tube to assess the end point dilution of specific cytotoxic activity.

RESULTS AND DISCUSSION

SERUM CYTOTOXIC AND OPSONIC CURVES DURING THE PRIMARY AND SECONDARY IMMUNE RESPONSE

Primary immune serum from a uniform group of C57Bl mice, injected with L5178Y cells, was collected at defined intervals, heat inactivated and tested for cytotoxicity and opsonic activity. 36 days after the first injection half of the mice remaining received a second i.p. injection of 10^7 L5178Y cells. Fig. 1 shows the curves for the primary response cytotoxic antibody and opsonic activity over a period of 50 days, and the inset shows the curves during the first 14 days of the secondary immune response. During the primary response opsonic activity was detected 2 days after injection of lymphoma cells and it increased to reach a peak by 10–14 days. Cytotoxic antibody was not detected until after 4 days but reached its peak activity at the same time as the opsonic. Both activities declined after 14 days. By 28 days cytotoxicity was not demonstrable in the serum, while opsonic

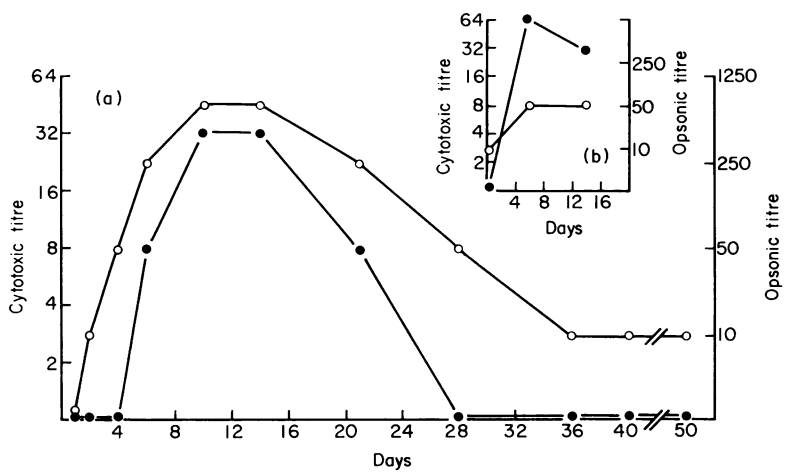

FIG. 1. Serum opsonic (○) and cytotoxic activity (●) of mice injected with L5178Y cells during (a) the primary immune response and (b) the secondary immune response.

activity remained at a low level for at least 50 days. The serum of mice receiving a second injection of lymphoma cells on day 36 showed a small increase in opsonic activity, but cytotoxic antibody reached a higher level than previously found in the primary response. Thus, although cytotoxic and opsonic curves appeared to run parallel during the primary response there was a difference in their times of appearance in the serum and subsequent disappearance. The relative concentration of opsonic activity, compared with cytotoxicity, during the secondary response was obviously much lower than that during the primary response, and corresponded to the level of cytotoxic activity.

In another series of experiments using serum which had not been heat inactivated, results comparable to those described above were obtained, demonstrating that thermolabile components, for example complement, were not required for phagocytosis of L5178Y cells.

TREATMENT OF SERUM SAMPLES WITH SATURATED AMMONIUM SULPHATE

Serum samples from the above experiment collected 4, 7, 10 and 14 days after lymphoma injection of mice were tested for cytotoxic and opsonic activity before and after ammonium sulphate precipitation. The results showed that all cytotoxicity and opsonic activity were precipitated without loss.

ELECTROPHORETIC FRACTIONATION

The γ-globulin and non-γ-globulin-containing fractions were tested for cytotoxic and opsonic activity and compared with non-fractionated serum. Table 1 shows that most of the opsonic activity and all of the cytotoxic activity were recovered in the γ-globulin fraction. At 1/250 the degree of opsonization was strong, but was quite weak at 1/1250 (slightly less than 1 per cent of the macrophages contained ingested lymphoma cells). Although the end point is given as 1/250 in the table, the actual reduction in the original level of opsonic activity was clearly less than indicated. A small part of the opsonic activity was found in the non-γ-globulin fraction, which contained β-globulins, albumins and other proteins.

TABLE 1

OPSONIC AND CYTOTOXIC ACTIVITIES OF 14-DAY ANTI-L5178Y SERUM AFTER FRACTIONATION BY ELECTROPHORESIS INTO γ-GLOBULIN AND NON-γ-GLOBULIN-CONTAINING FRACTIONS

Serum sample	Opsonic titre	Cytotoxic titre
Untreated immune serum	1/1250	1/50
γ-Globulin fraction	1/250	1/50
Non-γ-globulin fraction	1/10	<1
Normal serum	<1/2	<1

MERCAPTOETHANOL TREATMENT OF SERUM SAMPLES

Serum samples collected for ammonium sulphate precipitation on days 4, 7, 10 and 14 were treated with mercaptoethanol, as described under Experimental Procedures (f). All were assayed for cytotoxic and opsonic activity and compared with untreated sera (Table 2). It is seen that opsonic activity was completely abolished on day 4, and was reduced thereafter in all other samples to the same level as cytotoxic activity, which was not

TABLE 2

EFFECT OF MERCAPTOETHANOL-TREATMENT OF SERUM* COLLECTED VARIOUS DAYS AFTER INOCULATION OF MICE WITH L5178Y CELLS

	Opsonic titre		Cytotoxic titre	
Serum collected	Before	After	Before	After
Day 4	1/27	<1/3	<1/3	<1/3
Day 7	1/243	1/9	1/9	1/9
Day 10	1/729	1/27	1/27	1/27
Day 14	1/729	1/27	1/27	1/27

* Three-fold dilutions prepared in Fischer's medium.

affected by this treatment. These results suggest that the mercaptoethanol-resistant, complement-dependent activity is due to 7S-antibody, while the mercaptoethanol-sensitive opsonic activity is due to 19S-antibody. The time of appearance of both activities in the serum would be similar to the early appearance of 19S, followed later by 7S antibody in a number of different infections (Johnson, 1964; Svehag and Mandel, 1964; Ure and Finkelstein, 1963). The above results require that the cytotoxic antibody is also opsonic, although accounting for only a small proportion of the total opsonic activity.

BINDING OF CYTOTOXIC AND OPSONIC ACTIVITIES TO LYMPHOMA CELLS

In an attempt to absorb all specifically binding lymphoma antibodies, the effect on cytotoxic and opsonic activity was tested after absorbing anti-L5178Y serum with lymphoma cells. Normal and 14-day primary immune serum samples were tested before and after absorption. The supernatants recovered after thorough washing of the cells were also tested (see Experimental Procedures (g). Table 3 shows that a single absorption did not remove all cytotoxic or opsonic activity (a second absorption with fresh lymphoma cells completely removed cytotoxic antibody and reduced the opsonic activity). On washing the

TABLE 3

EFFECT ON CYTOTOXIC AND OPSONIC ACTIVITY OF ABSORBING 14-DAY ANTI-L5178Y SERUM WITH L5178Y CELLS*

Sample	Opsonic titre	Cytotoxic titre
Immune serum not absorbed	1/1250	1/50
Same diluted 1/5	1/250	1/10
Serum after absorption with cells	1/50	1/5
Pooled supernatants from washed cells	1/250	$<1/5$

* 1 ml of undiluted anti-serum absorbed with 10^8 L5178Y cells for 2 hours. Five-fold dilutions prepared.

cells, all of the opsonic activity was recovered in the supernatants, but this had no cytotoxic activity. That the reduction of cytotoxic antibody was due to binding to the lymphoma cells was shown by the addition of guinea-pig serum to the cells after absorption. This resulted in death of over 95 per cent of the cells. Lymphoma cells incubated in normal serum were not killed (less than 3 per cent were stained with trypan blue). From this experiment, it is clear that cytotoxic antibody of primary immune serum binds strongly to L5178Y cells, and is quite distinct from the non-binding opsonic activity, which appears to have little affinity for L5178Y cells.

A further experiment to test that cytotoxic antibody is also opsonic is described in Experimental Procedures (h). Duplicate dilution series of 14-day immune serum were mixed with L5178Y cells. One dilution series was thoroughly washed, the other not, and each individual dilution was tested for opsonization of the suspended lymphoma cells, and for the presence of cytotoxic activity. Table 4 shows that when L5178Y cells were washed after incubation in the dilutions of antiserum, opsonization occurred up to a dilution of 1/50, which was also the dilution end point for cytotoxic activity. If the cells were not washed, opsonic activity was found at 1/1250, but cytotoxicity did not occur beyond 1/50. This confirms the findings of the experiment shown in Table 2 that strongly binding cytotoxic antibody opsonizes L5178Y cells but forms only a small proportion of the total opsonic activity.

TABLE 4

OPSONIC ACTIVITY OF CYTOTOXIC ANTIBODY OF ANTI-L5178Y-SERUM

Treatment after incubation with antiserum	Dilution end point showing:	
	Opsonization	Cytotoxicity
L5178Y cells washed	1/50	1/50
L5178Y cells not washed	1/1250	1/50
Controls with or without normal serum	<1/2	<1/5

SPECIFICITY OF CYTOTOXIC ANTIBODY AND OPSONIC ACTIVITY

Further experiments to demonstrate differences between cytotoxic antibody and opsonic activity involved testing the extent, if any, of cytotoxic and opsonic cross-reactivity when L5178Y, TLX5 and sheep red blood cells were mixed with serial dilutions of the foreign antisera, as described in detail under Experimental Procedures (i). (In these experiments sera were not heat-inactivated.) The subsequent cytotoxic and opsonic tests revealed the extent of specificity of each antiserum (Table 5). It is seen that both anti-L5178Y and TLX5 sera cross-reacted opsonically with TLX5 and L5178Y cells respectively as long as the cells were not washed after the initial incubation. On washing the cells, opsonization of the foreign antigen was completely abolished. Both lymphoma antisera opsonized sheep red cells, but this effect was greatly reduced on washing. The cytotoxic effect of the lymphoma antisera was completely specific for the immunizing antigen. Anti-sheep red cell serum was different from the lymphoma antisera by being completely specific in its cytotoxic and opsonic properties. Furthermore, if heat-inactivated lymphoma

TABLE 5

CROSS REACTIVITY OF CYTOTOXIC AND OPSONIC ACTIVITY OF ANTI-L5178Y, TLX5 AND SHEEP RED BLOOD CELL ANTISERA* WITH L5178Y, TLX5 OR SHEEP RED BLOOD CELLS

Sample and treatment after incubation	End point showing:	
	Opsonization	Cytotoxicity
1. Anti-L5178Y serum:		
L5178Y cells not washed	1/1250	1/50
L5178Y cells washed	1/50	1/50
TLX5 cells not washed	1/50	<1/5
TLX5 cells washed	<1/2	<1/5
SRBC not washed	1/6250	<1/5
SRBC washed	1/50	<1/5
2. Anti-TLX5 serum:		
TLX5 cells not washed	1/250	1/50
TLX5 cells washed	1/50	1/50
L5178Y cells not washed	1/50	<1/5
L5178Y cells washed	<1/2	<1/5
SRBC not washed	1/6250	<1/5
SRBC washed	1/10	<1/5
3. Anti-sheep red blood cell serum:		
SRBC not washed	>1/6250	>1/6250
SRBC washed	>1/6250	>1/6250
L5178Y and TLX5 cells washed and unwashed	<1/2	<1/5

* Sera not heat-inactivated.

antisera were used, opsonization of sheep red cells did not occur, thus demonstrating the need for a thermolabile component for successful erythrophagocytosis, previously shown by Gigli and Nelson (1968). Since the use of heat-inactivated lymphoma antisera did not affect the ingestion of TLX5 or L5178Y cells, the mechanisms involved during phagocytosis of sheep red cells or lymphoma cells may be quite different. At present, it is not known whether the higher levels of opsonization of L5178Y and TLX5 cells in the presence of their respective antisera, as opposed to lower levels seen in the presence of foreign lymphoma antiserum, represents a low degree of specificity for the immunizing antigen. The fact that sheep cells were opsonized at higher levels than TLX5 or L5178Y cells by the lymphoma antisera, together with the report that a number of other animal red cells may be opsonized by normal serum factors (Vaughan, 1965) would agree with the suggestion that the mechanism of erythrophagocytosis may differ from that of tumour cell ingestion.

The data presented above clearly show that primary antiserum directed against L5178Y or TLX5 cells contains two distinct types of opsonic activity. One is associated with cytotoxic antibody and binds strongly to the appropriate lymphoma cells, while the other is not associated with cytotoxicity and may easily be eluted from the immunizing antigen. Both opsonic activities of anti-L5178Y serum were associated with γ-globulin fraction of serum, and on the basis of mercaptoethanol sensitivity may belong to the IgM class. Although lymphoma cytotoxic antibody was shown to be opsonic, thereby resembling 7S-anti-bacterial opsonic activity described by Laxdal *et al.* (1968), there is no evidence to suggest that the mechanisms of phagocytosis in the two sysems are the same. The tumour specific opsonic activity associated with cytotoxic antibody may well prove to be another method of measuring tumour specific cytotoxic antibody, as demonstrated by Bennett *et al.* (1963), who used hyperimmune anti-tumour sera in their experiments. However, these authors found that their sera were totally specific for the immunizing antigen. It would seem, therefore, that a feebly-binding opsonic activity was not present in hyperimmune sera, unless it occurred at a level too low to opsonize effectively foreign antigens. This is a possibility in view of a statement in a discussion of a paper by Bennett *et al.* (1964) that when tumour cells which had been exposed to specific allogeneic antisera were washed, 'phagocytosis was lessened in extent', suggesting the presence of non-binding opsonin. What the significance is of such opsonic activity and whether its appearance during the primary immune response is a typical reaction after tumour implantation requires further exploration. Experiments are currently under way to elucidate the role *in vivo* of strongly binding opsonins and those of low avidity during the reactions involved in the rejection of tumour homografts.

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