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Surface markers of small lymphocytes appearing in the mouse Ehrlich ascites tumour, host spleen and blood

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Summary. Small lymphocytes sampled from intraperitoneally growing Ehrlich ascites tumour in CBA/H-T_6 mice as well as host spleen and blood at different days of tumour development were characterized radioautographically on the basis of two surface markers, IgM for B cells and θ antigen for T cells. A direct binding of ^{125}I-labelled anti-IgM detected natural surface IgM, while an indirect binding following a prior exposure to anti-θ antibody detected θ antigen. Cells remaining unlabelled with the latter procedure were considered to lack both markers (double negative). While the incidence of IgM+ve small lymphocytes within the tumour declined, their absolute numbers increased with tumour growth. Low levels of antiglobulin binding shown by these cells were considered to reflect low levels of maturation, because (1) our previous studies indicated that they were newly formed, and (2) the extent of antiglobulin binding by B lymphocytes in the marrow is known to increase with increasing post-mitotic age. The proportions and the absolute numbers of θ+ve as well as the double negative small lymphocytes increased within the growing tumours. Within the host spleen, the incidence of IgM+ve small lymphocytes remained unchanged but their absolute numbers increased because of splenomegaly. The degree of antiglobulin binding by these cells was comparable to that of the normal splenic population. The incidence of θ+ve cells dropped but their absolute numbers remained unchanged in the spleen during tumour growth. In contrast, the incidence as well as the absolute numbers of double negative cells increased markedly. This cell category increased also in the blood, possibly in transit to the tumour site and other lymphoid organs from the bone marrow, where they were most prevalent. Their bone marrow origin was further suggested by a preponderance of marrow derived small lymphocytes at the tumour site as well as in the host spleens found in our earlier studies. Double negative population in the spleen showed a paucity of C'3 and Fc receptors on the cell surface and included cells capable of producing B lymphoid colonies *in vitro*.

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INTRODUCTION

Spontaneous as well as experimentally produced tumours often exhibit the presence of a variable number of host leucocytes, particularly lymphocytes, monocytes and macrophages. Despite the long-standing controversy over their role in host tumour relationships (Underwood, 1974), attempts to characterize these cells have only recently begun. *In vitro* functional potentials of the monocyte–macrophage class of cells isolated from tumours

have been evaluated by a number of investigators (Van Loveren & Den Otter, 1974; Haskill, Proctor & Yamamura, 1975; Holden, Haskill, Kirchner & Herberman, 1976). While surface markers and functional potentials of lymphocyte subpopulations have been examined in peripheral lymphoid organs such as lymph nodes (Loring & Schlesinger, 1970), spleen (Konda, Nakao & Smith, 1973; Kirchner, Chused, Herberman, Holden & Lavrin, 1974; Kirchner, Muchmore, Chused, Holden & Herberman, 1975; Dorizzi, Ortiz-Muniz, Lopez, Sigel & Epstein, 1975) or peripheral blood (Malka, Oon & Hobbs, 1974; and many others) of tumour bearing hosts, lymphocytes invading tumours have not been sufficiently characterized. A number of studies have provided only a partial picture, because they were confined to a single surface marker (Cardozo & Harting, 1972; Blomgren, Glas, Franzen & Granberg, 1973; Nind, Nairn, Rolland, Guli & Hughes, 1973; Kerbel, Pross & Elliott, 1975; Djeu, McCoy, Cannon, Reeves, West & Herberman, 1976; Szymaniec & James, 1976). More definitive studies on some murine tumours include an examination of surface properties of local mononuclear leucocytes showing specific *in vitro* cytostatic (Haskill, Yamamura & Radov, 1975) or cytotoxic (Holden *et al.*, 1976) effects on tumour cells, and a morphological localization of two lymphocyte surface markers: Ig on B cells and brain associated thymus antigen on T cells by immunofluorescent staining (Russell, Gillespie, Hansen & Cochrane, 1976).

Previous studies in this laboratory revealed that there was a selective migration of newly formed lymphocytes from the circulation into intraperitoneally growing Ehrlich ascites tumour (Lala, 1974) or subcutaneously growing TA-3(St) line tumour in mice (Kaizer & Lala, 1977); a large majority of these cells were found to be marrow derived (Lala, 1976). Furthermore, tumour transplantation was found to cause a rapid splenomegaly, primarily attributed to an increase in the size of the small lymphocyte population which, in turn, resulted from extraneous migration as well as local lymphoid proliferation within the spleen (Lala, Terrin, Lind & Kaizer, 1977). With the use of chromosome markers in this study, the majority of the cells proliferating in the enlarged spleen, or their immediate precursors, were also identified as bone marrow derived. Thus, it was conceivable that many of the newly formed small lymphocytes migrating into the Ehrlich ascites tumour or accumulating in the host spleen might represent relatively immature B cells. For this reason, the present study was designed in part to examine the maturation level of IgM-bearing B lymphocytes in these tissues as reflected by the degree of anti-IgM binding in radioautographic preparations. This approach was based on the studies by Osmond & Nossal (1974b), who combined ^{3}H-thymidine labelling as a marker of cell age with radioiodinated anti-IgM binding as a probe for surface IgM, to reveal that small lymphocytes in the normal murine bone marrow did not show detectable surface binding of anti-IgM when first formed; following a post-mitotic lag of 1·5 days, they exhibited detectable antiglobulin binding, the extent of which increased with increasing post-mitotic age. In the second part of this study, the composition of small lymphocyte populations appearing in the Ehrlich ascites tumour, as well as in the blood and the spleen of the tumour bearing hosts at various stages of tumour development was examined radioautographically with respect to two surface markers, IgM on B cells and θ antigen on T cells. For this purpose, we employed an indirect (sandwich) immunolabelling protocol devised in this laboratory. The findings were then related to the existing knowledge of their dynamics and life history.

MATERIALS AND METHODS

Animals

CBA/H-T_6 strain female mice (Jackson Laboratories) at 12–15 weeks-of-age were used throughout this study.

Tumour

Ehrlich ascites tumour, obtained from Dr T. Hauschka (Roswell Park Memorial Institute) 12 years ago, was initially maintained in CF_1 strain mice by a weekly intraperitoneal transfer of 10^6 tumour cells. Another subline used in the present study was established from this original tumour about 4 years ago by transfer into CBA/H-T_6 strain female mice and maintained in a similar fashion within this strain. The tumour has a near-tetraploid amount of DNA in post-mitotic cells, and a modal chromosome number of 68.

Tumour growth was measured according to Lala & Patt (1966). The total number of various leucocyte types present within the ascites tumours at

various days of growth was computed from differential counts of MacNeal's tetrachrome stained smears, applied to the total tumour cell number. A minimum of 500 leucocytes and associated tumour cells were scored. Leucocytes recovered from peritoneal lavage of normal, tumour-free animals provided control (0 day) values.

Preparation of cell suspensions for surface marker studies

Single cell suspensions were prepared in the cold according to the technique employed by Osmond & Nossal (1974a) from the following tissues: femoral bone marrow and thymus of normal animals, and spleen and cardiac blood of normal as well as tumour bearing animals. Blood was subjected to erythrocyte lysis with buffered hypotonic NH_4Cl solution prior to use. Cells were finally suspended in ice-cold HEPES-buffered Eagle's minimal essential medium containing 10% foetal calf serum (10% HEM-FCS; both materials obtained from Grand Island Biological Company, N.Y.) at a concentration of 20×10^6 nucleated cells/ml. Normal peritoneal leucocytes as well as cell suspensions from ascites tumours (inclusive of host-derived leucocytes) were washed in an excess of ice-cold 10% HEM-FCS prior to their resuspension at a similar nucleated cell concentration.

Sera

The 7S IgG fraction of an anti-mouse IgM (anti-μ) serum, raised in goats, was obtained from Meloy Laboratories, Springfield, Va., U.S.A. The anti-IgM was monospecific in immunoelectrophoresis tests. Anti-θ serum (*Thy.* 1.2, AKR anti-C_3H) was obtained from Litton Bionetics Inc., Kensington, Maryland, U.S.A.; this was cytotoxic to >90% C_3H or CBA/H-T_6 thymocytes at 1/100 dilution. Key results from immunolabelling experiments (described later) using this serum were confirmed with a high potency *Thy.* 1.2 antiserum raised in congenic mice by Jackson Laboratories (kindly provided by Dr J. G. Ray, Transplantation and Immunology Branch, National Institutes of Health, Bethesda, Md.). Normal mouse serum was obtained from healthy, adult AKR or C57Bl mice. All sera were inactivated at 56° for 30 min before use in surface marker studies.

Radioiodination of antiserum

For each experiment, an aliquot of 260 μg of anti-IgM protein was labelled with 2·6 mCi of carrier-free $Na^{125}I$ without preservatives (Charles E. Frosst & Co., Kirkland, Quebec, Canada) by a modification of the chloramine-T oxidation method of Greenwood, Hunter & Glover (1963). Free iodide was removed by passage of the labelled protein solution through a Sephadex G-25 fine column (Pharmacia (Canada) Ltd., Dorval, Quebec). The resulting specific activity of the ^{125}I-labelled anti-IgM ranged from 5 to 6 μCi/μg of protein.

Direct labelling of the cells with ^{125}I-anti-IgM

This was performed according to the method of Osmond & Nossal (1974a). In brief, 0·1 ml aliquots of each cell suspension containing 2×10^6 nucleated cells were incubated for 30 min at 0° with equal volumes of ^{125}I-labelled anti-IgM at different final antiglobulin concentrations: 1, 3, 10 and 20 μg/ml. Cells were then washed twice by centrifugation at 400 *g* for 7 min at 4° through 3 ml discontinuous gradients of FCS (100, 75 and 50%). The final pellets were then resuspended in a minute amount of 100% FCS for smearing on slides previously coated with 0·5% gelatin containing 0·05% chrome alum.

Indirect (sandwich) labelling of θ antigen bearing lymphocytes with ^{125}I-anti-IgM

The basis of the technique was identical to that reported by Nossal, Warner, Lewis & Sprent (1972) for other receptors. Aliquots (0·1 ml containing 2×10^6 nucleated cells) from each tissue suspension were mixed with 0·1 ml of normal mouse serum at a dilution of 1 : 20. These constituted the 'Control serum-treated' group for the experiment. Identical aliquots were mixed with 0·1 ml of anti-θ serum to give a final dilution of 1 : 20. These constituted the 'anti-θ serum-treated' group. Cell suspensions in both groups were incubated for 30 min at 0°. They were then spun through discontinuous gradients of FCS (as mentioned earlier) to remove any unbound serum. The cell pellets in both groups were then resuspended in 0·1 ml of 10% HEM-FCS and exposed to 0·1 ml of ^{125}I-labelled anti-IgM at a final concentration of 10 μg/ml for 30 min in the cold. All further procedures after this step were identical to those described in the direct immunolabelling procedure.

Radioautographic analysis of materials from 'control serum-treated' group in pilot experiments revealed identical labelling values compared to those obtained with the direct labelling technique, where

the normal (AKR or C57Bl) serum step was omitted. Thus, labelling indices in this series provided the incidence of IgM+ve cells; additional labelling in the anti-θ serum treated series provided the incidence of θ+ve cells. The concentration of anti-θ antibody used above was higher than needed to saturate θ antigenic sites, and thus identify all θ antigen bearing cells with the present protocol. For example, a maximal plateau labelling (92–95%) for small lymphocytes in the thymus was achieved with the sandwich labelling technique using anti-θ serum dilutions ranging between 1/80 and 1/10. Similarly, the final concentration of radiolabelled anti-IgM was decided on the basis of pilot experiments using concentrations ranging between 1 and 20 μg/ml in the sandwich labelling technique. Maximal labelling (93–95%) of small lymphocytes in the thymus was attained at concentrations $\geqslant 3$ μg/ml.

Radioautography

Smears fixed in absolute methanol were dipped in Kodak NTB2 liquid emulsion diluted with an equal volume of distilled water, and exposed for 4 days at 4°. After developing, the smears were stained with Giemsa.

Analysis of radioautographs

Labelling indices were based on scoring 400–2000 small lymphocytes (and other associated leucocytes on most occasions) along the longitudinal mid-axis of each radioautograph. In one experiment, a detailed grain count on small lymphocytes sampled from the spleens of normal control animals and 7 day old ascites tumours was performed for different antiglobulin concentrations employed in the direct immunolabelling technique.

Morphological criteria used for identifying lymphocytes were those employed by Osmond & Nossal (1974a). Further subdivision of lymphocytes into different size categories was done from the measurement of nuclear diameters as follows: small (8 μm or less), medium ($>8<11$ μm), and large ($>11<14$ μm). Most tumour cells had large round or oval nuclei (minimum diameter 15 μm; average 25 μm) with very distinct nucleoli varying in number from 1 to 4. The cytoplasm was basophilic with a high nucleus to cytoplasmic ratio. An additional distinctive characteristic was the presence of circumferential cytoplasmic blebs. Lymphocytes as well as tumour cells were distinguishable from the monocyte–macrophage series of cells on the basis of morphological criteria reported earlier (Kaizer & Lala, 1977; Lala & Kaizer, 1977) for Giemsa stained smears, correlated with cytochemical criteria for enzyme markers. For example, most monocytes were weakly peroxidase positive (van Furth, Hirsch & Fedorko, 1970), and most macrophages were nonspecific esterase positive (Yam, Li & Crosby, 1971).

The threshold number of silver grains for positive labelling was determined according to the method of Lala & Patt (1966), based on the distribution of background grain numbers. The threshold varied under different conditions, such as the antiglobulin concentration used, or the type of tissue employed. For example, the average threshold for small lymphocytes within the spleen, blood and ascites tumour was 20, 6 and 4 grains respectively, at an antiglobulin concentration of 10 μg/ml.

RESULTS

Tumour growth curve

Fig. 1 represents the number of various cell types recovered from the ascites fluid at various days of Ehrlich ascites tumour development. Amongst host-derived leucocytes, lymphocytes constituted the highest proportion at earlier intervals. Macrophage numbers, although initially rather small, increased subsequently to match the monocyte numbers, and finally exceeded all other leucocyte numbers in older tumours. Granulocytes constituted a minor proportion throughout the first week of tumour growth, but increased during the second week (not shown in the figure). Present studies on lymphocyte surface markers were confined to the first week.

Dose response for detectable anti-IgM binding by small lymphocytes

With the direct immunolabelling technique, labelling indices of normal splenic small lymphocytes reached a plateau level at antiglobulin concentrations of 3 μg/ml or higher, indicating that under these conditions all mature splenic small lymphocytes with readily detectable surface IgM were being identified (Fig. 2). Consequently, in further experiments designed to estimate the incidence of IgM+ve cells using a single antiglobulin concentration, 10 μg/ml was used; at higher concentrations the background number of grains increased significantly. Within the

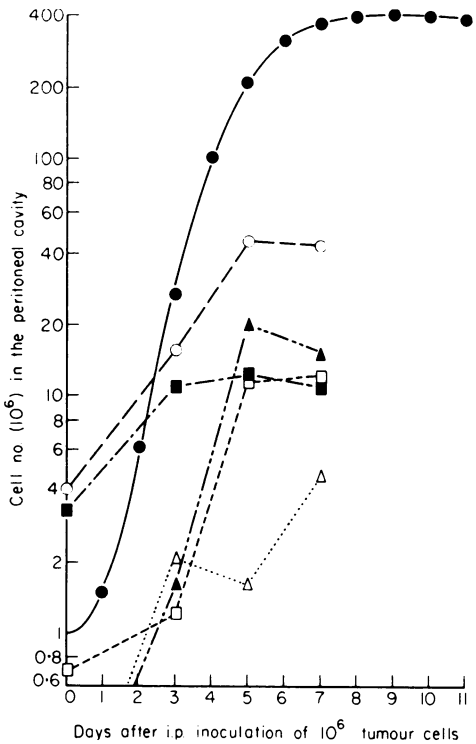

Figure 1. Absolute number of various cell types within the Ehrlich ascites tumours at different days of growth. Zero day values for leucocytes represent peritoneal cell populations in normal mice. Each point represents the mean value from three animals. (●) Tumour cells; (○) total leucocyte; (▲) macrophage; (□) monocyte; (■) total lymphoid; (△) granulocyte.

spleens of 7 day old tumour-bearing hosts, small lymphocyte labelling indices were slightly lower than the values in the normal spleen at all antiglobulin concentrations, but the differences were not statistically significant at the 0·05 level, and the labelling patterns were fairly parallel. In contrast to the picture in the spleens, the percentage of labelled small lymphocytes sampled from the 7 day-old ascites tumours increased steadily with increasing antiglobulin concentrations up to 20 μg/ml with no indication of a labelling plateau. This indicated that many of these cells could bind anti-IgM only at high anti-IgM concentrations. Relatively low levels of anti-IgM binding by these cells were further substantiated from a detailed grain count done in one set of experiments.

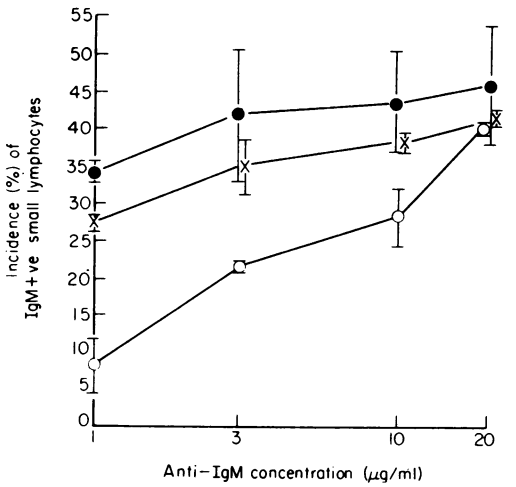

Figure 2. Anti-IgM binding by small lymphocytes from normal spleens, the spleens of mice bearing the 7 day old Ehrlich ascites tumour, and the 7 day old tumours themselves. The direct immunolabelling technique was employed at various anti-IgM concentrations. Each point represents the mean of two experiments (vertical bars indicating ranges) and tissues from two animals were pooled in each experiment. (●) Normal spleen; (×) spleen of 7 day tumour host; (○) 7 day tumour.

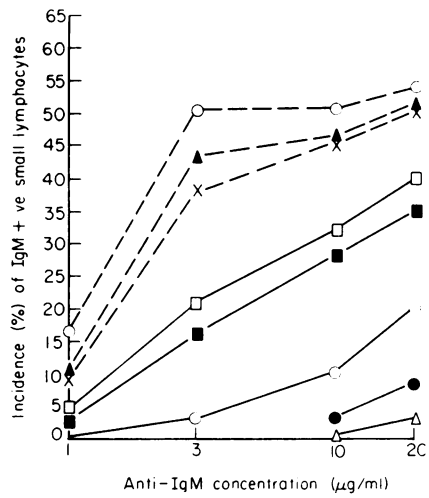

Figure 3. Small lymphocyte labelling indices in normal spleens and in 7 day old Ehrlich ascites tumours, exposed to different concentrations of ^{125}I-anti-IgM, at various grain thresholds, illustrating results of one experimental set in which tissues from two animals were pooled. (○- - -○) 20^+ grains normal spleen; (▲- - -▲) 40^+ grains normal spleen; (×- - -×) 50^+ grains normal spleen; (□——□) 4^+ grains 7 day tumour; (■——■) 5^+ grains 7 day tumour; (○——○) 10^+ grains 7 day tumour; (●——●) 20^+ grains 7 day tumour; (△——△) 40^+ grains 7 day tumour.

Labelling intensity of small lymphocytes in the normal spleen and in the 7 day old Ehrlich ascites tumour

The number of silver grains associated with small lymphocytes were scored in one experiment at all antiglobulin concentrations employed. These data were then used to plot the incidence of labelled small lymphocytes at various grain thresholds (Fig. 3). In the normal spleen, the labelling index increased at all grain threshold levels between concentrations of 1 and 3 μg/ml. At higher concentrations, there was little or no further increase of cells with 20 or more grains. However, cells with more than 40 or 50 grains increased slightly. In this case, 20 grains were taken as a threshold for labelling at concentrations of 3 μg/ml or higher. For the small lymphocytes within the 7 day tumour, labelling indices increased with increasing anti-IgM concentrations at all grain thresholds. Cells with 4 or more grains were considered as labelled in this instance. In contrast to the normal spleen, cells showing 40 or more grains were rare within the tumour even at the highest anti-IgM concentration. The weak labelling exhibited by these cells appeared to be due to specific anti-IgM binding, since this labelling was found to be blocked by a prior incubation of cells with the unlabelled 7S component of goat anti-mouse IgM but not with an identical concentration of 7S component of normal goat serum.

Relative incidence of IgM+ve, θ+ve and 'double negative' small lymphocytes in normal lymphoid tissues

Table 1 presents the results of sandwich immunolabelling technique applied to several normal lymphoid organs. The values compare favourably with the range of data published in the literature using different techniques, also included in this table. The incidence of double negative small lymphocytes was found to be highest in the bone

Table 1. Incidence (%, including ranges) of small lymphocyte subsets in normal mouse tissues in the present study, as compared to selected data reported in the literature for conventional mouse strains

Tissue	Surface marker +ve		Double negative	Technique*	Reference
	B	T			
Bone marrow	47	7	46	Present	Present
	47	Negligible	Rest	1,2	Osmond & Nossal (1974a)
	34	8	—	3,4	Ryser & Vassalli (1974)
	—	4	—	1	Röpke *et al.* (1975)
	39	0	—	5,2	Raff (1971)
Thymus	4	90	6	Present	Present
	(3–4)	(88–91)	(5–8)		
	3·6	—	—	1	Osmond & Nossal (1974a)
	—	94–100	—	2	Röpke *et al.* (1975)
	0	100	0	5,2	Raff (1971)
Spleen	46	41	13	Present	Present
	(37–53)	(33–49)	(10–14)		
	48	38†	14†	1,2	Osmond & Nossal (1974a)
	29–51	39–46	7–12	6	Stobo *et al.* (1973)
	64	30	6–8	1,7	Sidman & Unanue (1975)
	56	31	8	5,2	Raff (1971)
Blood	35	49	16	Present	Present
	29	—	—	1	Osmond & Nossal (1974a)
	33	71	6	5,2	Raff (1971)
Peritoneal space	59	23	18	Present	Present
	(58–60)	(19–27)	(15–21)		
	—	25–30	—	2	Raff (1971)

* Techniques employed: (1) antimouse IgM binding; (2) anti-θ cytotoxicity; (3) anti-MBLA (mouse B lymphocyte antigen) binding; (4) anti-MSLA (mouse small lymphocyte antigen) binding; (5) anti-MBLA cytotoxicity; (6) anti-κ and anti-θ binding (sandwich labelling); (7) anti-θ binding.

† Calculated from Osmond & Nossal (1974a) by the present authors.

marrow. Small but significant incidences of double negative cells were also found in the spleen, blood, the peritoneal space, and to a minor extent in the thymus.

Surface markers of small lymphocytes appearing within ascites tumours

As shown in Fig. 4, the relative incidence of IgM + ve small lymphocytes within the peritoneal cavity declined steadily, e.g. to approximately half of the

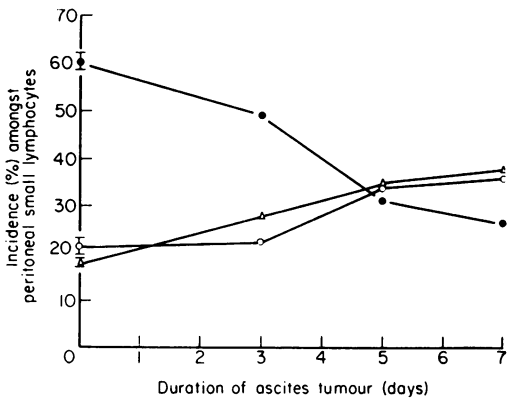

Figure 4. Incidence of IgM-bearing, θ antigen-bearing, and 'double negative' small lymphocytes in the normal peritoneal space (zero day) and in the Ehrlich ascites tumours of different ages. Cells from two animals were pooled in each experiment. (△) θ + ve; (○) double − ve; (●) IgM + ve.

0 day value at 7 days of tumour growth. Furthermore, the extent of antiglobulin binding by the IgM + ve cells also appeared to decline with increasing tumour age, as indicated by the increasing discrepancy between the proportions of IgM + ve cells detectable at two different antiglobulin concentrations (Table 2). The proportion of θ + ve as well as double negative small lymphocytes increased steadily to 38 and 36% respectively (Fig. 4).

The absolute numbers of the small lymphocyte subsets in the peritoneal cavity at different days of tumour growth calculated from data in Fig. 4 and the total small lymphocyte counts are presented in Fig. 5. A major increase in the number of all subpopulations was seen during the first 3 days of tumour growth, following which the patterns were different. While the total number of small lymphocytes showed further increase, reaching a maximum at day 5, the number of IgM + ve small lymphocytes declined between 3 and 7 days. Both the θ + ve and the double negative populations increased and then reached a plateau between 5 and 7 days. The 7 day values, compared to the 0 day values, were 1·5 fold for the IgM + ve cells, 7 fold for the θ + ve cells, and 6 fold for the double negative cells.

Surface markers of splenic small lymphocytes at different days of tumour growth

As shown in Fig. 6, the proportion of IgM + ve small lymphocytes in the spleen remained essentially unchanged, showing an insignificant decline at later intervals of tumour growth. The incidence of θ + ve small lymphocytes declined to approximately half of the 0 day value at 3 days and then showed minor fluctuations. The incidence of double negative cells doubled at 3 days and remained level thereafter.

Spleen sizes in these animals increased markedly during Ehrlich ascites tumour development, and during the first week lymphoid cells accounted for

Table 2. Labelling indices (%) of small lymphocytes in ascites tumours of different ages at two antiglobulin concentrations in the 'control serum treated' series

Tumour age	^{125}I-anti IgM conc. 1 μg/ml (a)	^{125}I-anti IgM conc. 10 μg/ml (b)	% discrepancy $\left(\frac{b-a}{b} \times 100\right)$
0*	59	62	4·9
3	29	49	40·8
5	17	31	45·2
7	13	26	50

* Normal peritoneal lymphocytes.

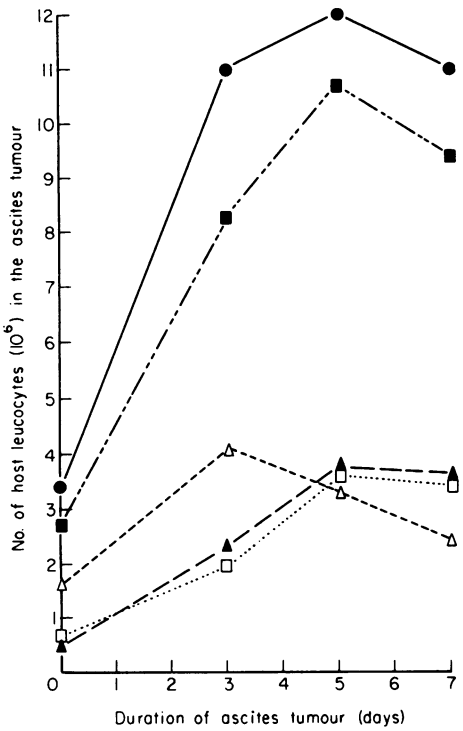

Figure 5. Absolute numbers of various lymphocyte subpopulations within the normal peritoneal cavity (zero day) and within the developing Ehrlich ascites tumours at different days of growth. (●——●) Total lymphoid; (■—·—■) total small lymphocytes; (▲– – –▲) small lymphocytes θ+ve; (□. . .□) double −ve; (△– – –△) IgM +ve.

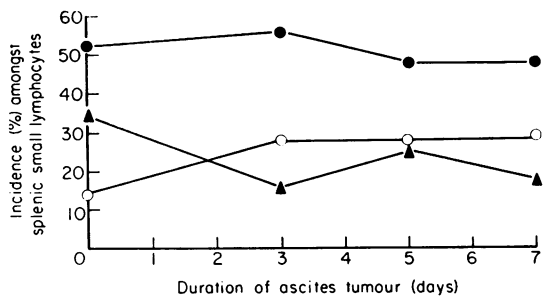

Figure 6. Incidence of IgM-bearing, θ antigen-bearing, and 'double negative' small lymphocytes in the normal spleens (zero day) and spleens of hosts at various days of Ehrlich ascites tumour growth. Tissues from 2 animals were pooled in each experiment. (●) IgM +ve; (○) double −ve; (▲) θ +ve.

most of this increase (Lala *et al.*, 1977). No tumour cells were observed in the spleen during this interval. The absolute numbers of the various small lymphocyte types in these spleens, calculated from their relative incidence (Fig. 5) and the total splenic small lymphocyte counts, are shown in Fig. 7. The total number of small lymphocytes doubled between 0 and 4 days and then remained level up to day 7. The sizes of IgM+ve as well as double negative small lymphocyte populations increased continuously; the former increased by a factor of 2 and the latter by a factor of 4, at 7 days of tumour growth. In contrast, the size of the θ+ve population remained virtually unchanged.

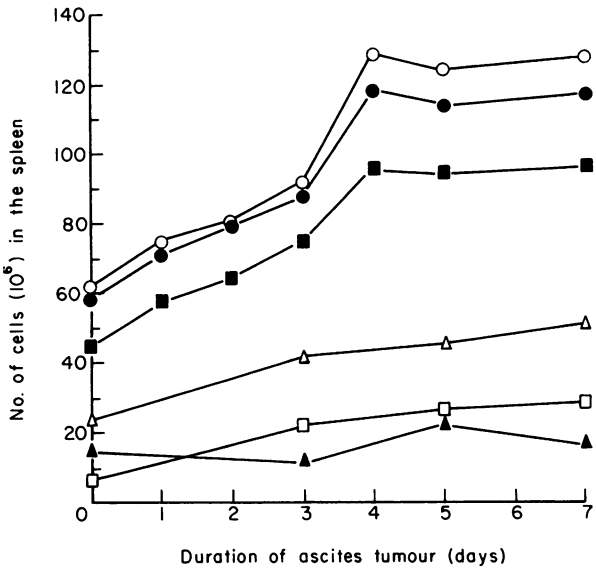

Figure 7. Absolute numbers of the total nucleated cells and the various lymphocyte types in the host spleens at various stages of Ehrlich ascites tumour development. (○) Total nucleated; (●) total lymphoid; (■) small lymphocyte; (△) IgM +ve small lymphocyte; (□) double −ve small lymphocyte; (▲) θ +ve small lymphocyte.

Surface markers of circulating small lymphocytes at different days of tumour growth

As shown in Fig. 8, the proportion of IgM+ve small lymphocytes declined from 34% in normal to 24% at 7 days of tumour growth. This was compensated for by an increase in the proportion of double negative cells. The incidence of θ+ve cells remained essentially unchanged at approximately 48%. The absolute number of lymphocytes in the blood dropped to about 50% of the 0 day value at day 3, possibly due to non-specific stress after

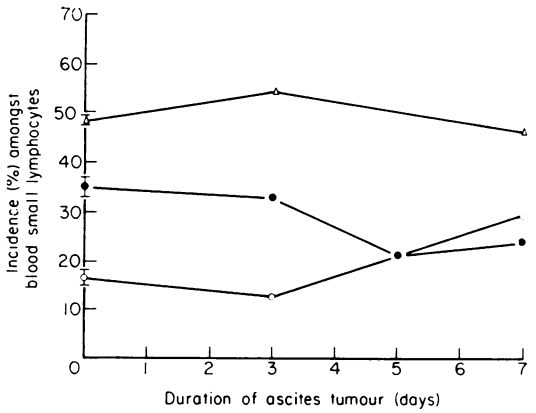

Figure 8. Incidence of IgM-bearing, θ antigen-bearing, and 'double negative' small lymphocytes in the blood of normal mice (zero day) and in the blood of hosts at various days of Ehrlich ascites tumour growth. (△) θ + ve; (○) double − ve (●) IgM + ve.

tumour transplantation (Lala *et al.*, 1977), followed by a complete recovery by day 4. Thus, their transient depletion affected all lymphocyte subsets, following which their absolute numbers behaved parallel to their proportions in the blood.

Labelling of cells other than small lymphocytes with ^{125}I-anti-IgM

Several observations, although not belonging to the mainstream of this study, deserve attention. Firstly, lymphocytes with diameters larger than 8 μm within the 7 day tumour as well as the spleen of 7 day tumour bearing mice showed a similar incidence of anti-IgM binding cells as noted for small lymphocytes at all antiglobulin concentrations. However, in the case of normal spleen, consistently higher proportions of IgM bearing cells were noted within the large lymphocyte class. Secondly, all cells in the monocyte–macrophage category within the ascites tumour showed anti-IgM binding owing to cytophilia for Ig molecules possibly via Fc receptors; such binding was higher for macrophages than for monocytes. Thirdly, ascites tumour cells showed considerable labelling with ^{125}I-anti-IgM, which could be appreciably reduced by a prior incubation of cells at 37° in protein-free medium for 1–2 h. This indicated that the labelling was due to a prior binding of immunoglobulins from the ascites fluid. Curiously, the binding of IgG molecules was found to be more labile than that of IgM molecules in this case, suggesting mechanisms other than Fc receptors (Lala, Garnis & Rahil, unpublished data).

Fig. 9 shows some radioautographs from the various labelling protocols.

DISCUSSION

Surface IgM on most IgM-bearing small lymphocytes within the advanced ascites tumour was detectable only at very high antiglobulin concentrations. Low levels of antiglobulin binding by these cells were further confirmed from grain counts. Whether the extent of antiglobulin binding revealed by the present technique provides a measure of surface receptor (IgM) density or some other phenomenon such as binding via Fc receptors may be an open question. Nevertheless, the present findings may be interpreted in two ways: (1) that the heterogeneity in antiglobulin binding by small lymphocytes within the tumour reflected a heterogeneity in maturation level along the B-cell pathway, as described for small lymphocytes in the normal murine bone marrow by Osmond & Nossal (1974b), or (2) that the B lymphocytes within the ascites tumour were 'specific' antigen-responders, whose receptors had been blocked by the antigens shed from the tumour or stripped off due to capping and endocytosis of receptor-antigen complexes. The former possibility is consistent with our previous studies which revealed that small lymphocytes accumulating within this tumour are predominantly newly formed cells (Lala, 1974), a large majority of which are marrow derived (Lala, 1976). The latter possibility is unlikely because of two reasons. Firstly, we found no change in the degree of anti-IgM binding by these small lymphocytes after incubation and repeated washes at 37° in large volumes of protein-free medium for periods up to 7 h, which should allow some receptor turnover. In contrast, this procedure substantially reduced the labelling of tumour cells themselves even when carried out for 1–2 h. This procedure also possibly excluded other mechanisms of dissociable Ig binding reported for some lymphocytes (Lobo, Westervelt & Horwitz, 1975). Secondly, no local lymphocyte proliferation, which might indicate a clonal expansion of antigen responsive cells, was observed within the tumour (Lala, 1974). Since the frequency of heavily labelled IgM + ve small lymphocytes was low and the

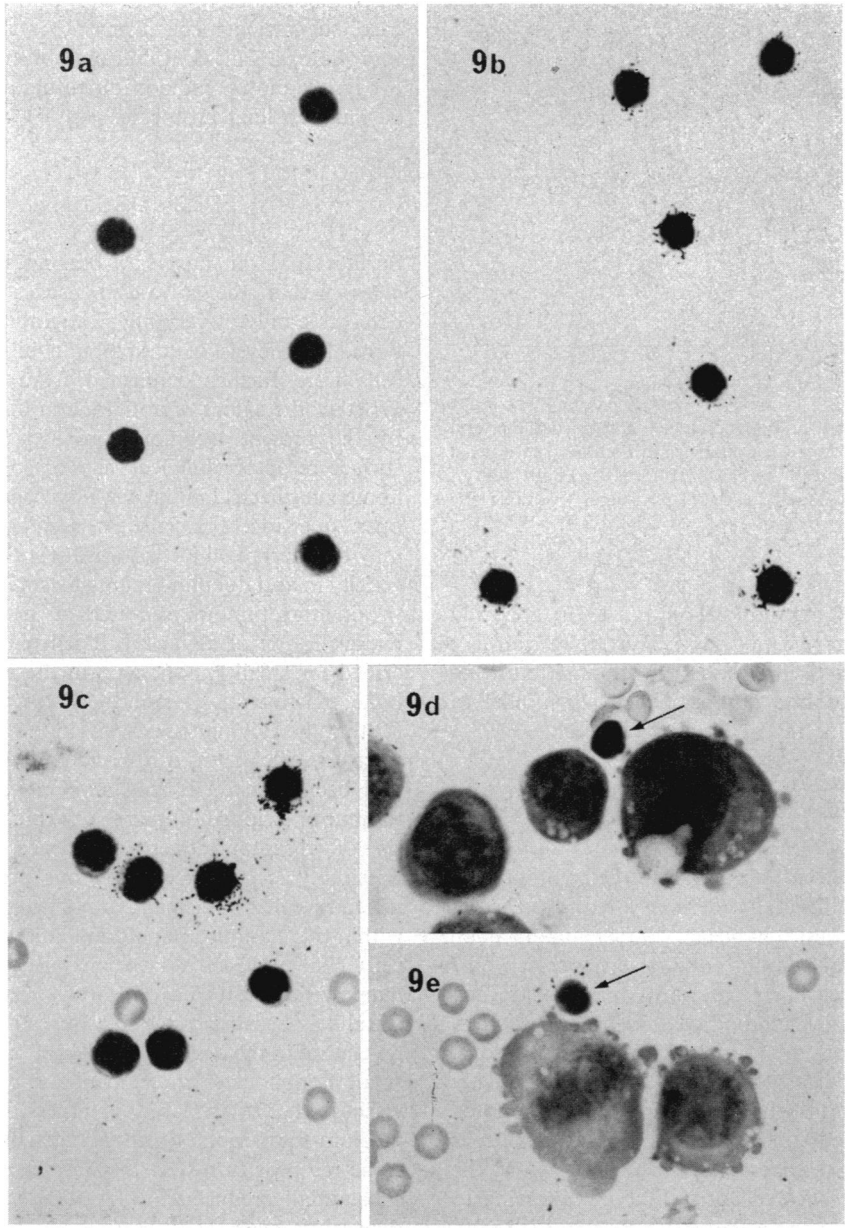

Figure 9. Selected radioautographs of various tissue preparations subjected to the indirect immunolabelling technique. (a) Normal thymic small lymphocytes from the control serum-treated series; none of the cells in the field is labelled. (b) Normal thymic small lymphocytes from the anti-θ serum-treated series; all cells in the field are labelled. (c) Splenic small lymphocytes from the 7 day tumour bearing host in the anti-θ serum-treated series, showing four labelled cells (including one weakly labelled) and three unlabelled (double negative) cells. (d) Seven day old Ehrlich ascites tumour from the anti-θ serum-treated series showing an unlabelled (double negative) small lymphocyte indicated by arrow. (e) Seven day old Ehrlich ascites tumour from the control serum-treated series showing a weakly labelled (IgM + ve) small lymphocyte indicated by arrow (all magnifications are × 1000).

occurrence of plasma cells was rare in this tumour at any age, it appeared that most B cells within this tumour were not yet fully equipped to mediate humoral immune responses. Within the spleens of the tumour-bearing animals, however, the IgM-bearing small lymphocytes exhibited comparable levels of anti-IgM binding, and hence presumably comparable maturation levels.

Several immunolabelling methods for detection of θ antigen have been reported in the literature (Stobo, Rosenthal & Paul, 1973; Sidman & Unanue, 1975; Aoki, Hämmerling, de Harven, Boyse & Old, 1969; Ben-Yaakov & Haran-Ghera, 1975; Bernard, Jeannesson, Zagury, Fridman, Ternynck & Avrameas, 1975; Cohen & Patterson, 1975). The indirect technique employed in the present study deserves several comments: (1) The success of the use of ^{125}I-anti-IgM as a final probe was based on the finding that most of the antigen-binding activity of the anti-θ serum appears to reside in the IgM fraction (Gershwin, Chused & Steinberg, 1974). (2) The technique was found to be highly sensitive. 'Double negative' cells detectable by this method were either truly double negative, or cells with extremely low density receptors (IgM or θ antigen), compared to peripheral B or T cells, since both the anti-θ and the anti-IgM antibody concentrations employed were much higher than needed to attain the maximal incidence of labelled lymphocytes in the normal spleen or thymus. Furthermore, increasing the duration of radioautographic exposure did not significantly change the proportion of double negative cells.

The present study revealed that the dynamics of various lymphocyte subsets in the peripheral lymphoid tissues during tumour development did not reflect the local picture at the tumour site, i.e. the peritoneal cavity. The incidence of IgM+ve cells declined markedly at the tumour site, moderately in the blood, but very little in the spleen. The proportion of θ+ve cells increased at the tumour site, showed little change in the blood, but declined in the spleen. The only common feature was a substantial increase in the proportion of double negative cells at all the three sites. The absolute number of all the three lymphocyte subsets, the θ+ve and double negative cells in particular, increased at the tumour site; but within the spleen the size of the θ+ve population remained the same, while others increased, and within the blood the increase was shown by the double negative class.

These findings may be considered in the light of previous observations in this laboratory of a selective increase in the incidence of newly formed (Lala, 1974) and marrow derived (Lala, 1976) small lymphocytes, within the developing tumour, and to a lesser extent, also in the spleen (Lala *et al.*, 1977) during the splenic enlargement after tumour transplantation. At the tumour site, the IgM+ve small lymphocytes, although having maturation levels consistent with a relatively short post-mitotic age, could account only in part for the marrow-derived lymphocytes, since the incidence of the latter increased with time, while that of the former actually decreased. The balance was most likely represented by the double negative population. Similarly, one has to include the double negative cells in the spleen of tumour-bearing animals amongst the marrow-derived class to account for an increase in their incidence (Lala *et al.*, 1977), since the incidence of IgM+ve cells did not change. A rise in the incidence of double negative cells in the blood possibly indicated their transit from the marrow to the tumour site as well as peripheral lymphoid tissues. Their presumed marrow origin is supported by the following: bone marrow is the only primary site of large scale lymphocyte production, which contains a large proportion of double negative small lymphocytes. Osmond & Nossal (1974b) observed that this population included early post-mitotic small lymphocytes of B cell lineage before they exhibited detectable surface IgM. However, some of these cells may not acquire surface IgM or other receptors (e.g. C'3 and Fc) *in situ* prior to their release from the bone marrow, and a further maturation, as indicated by receptor development, may occur in peripheral lymphoid organs (Osmond, personal communication). Alternately, they may remain 'null' for long periods (Stobo *et al.*, 1973) even in the peripheral lymphoid organs.

Double negative small lymphocytes within the spleen of tumour bearing mice have been further characterized by us to some extent. We have combined the sandwich radioimmunolabelling technique (for surface IgM and θ antigen) with the sheep erythrocyte rosetting technique of Parish & Hayward (1974a) for C'3 or Fc receptor to examine the presence of three receptors simultaneously (Lala, Garnis, Clavier & Jacobs, unpublished data). Results so far indicate a paucity of these receptors on most double negative cells. Thus, they cannot be equated with 'K' cells (mediators of antibody dependent

cytotoxicity), which are double negative but have Fc receptors (Greenberg, Hudson, Shen & Roitt, 1973; Ramshaw & Parish, 1976). The relationship of the present cells to natural killer ('NK') cells, which also lack B or T cell markers (Herberman, Nunn & Lavrin, 1975) is presently unknown. Two sets of evidence strongly suggest that splenic double negative lymphocytes in the present tumour-bearing mice include cells which can develop into B lymphocytes: (1) We have found a rapid rise in the incidence of B lymphoid colony forming cells (assayed after Metcalf, Nossa, Warner, Miller, Mandel, Layton & Gutman, 1975) in the spleen of tumour transplanted mice reaching a maximal level at day 3, the temporal pattern being very similar to that seen for double negative cells (Keeb & Lala, in press). This happened in the absence of a rise in the proportion of IgM-bearing cells as shown in the present study. (2) Lymphocytes from these spleens were exposed to anti-θ antibody and then rosetted with sheep erythrocytes coupled with anti-mouse IgM to rosette IgM as well as θ-bearing cells; exclusion of these rosettes by centrifugation through Ficoll–Hypaque gradients (after Parish & Hayward, 1974b) resulted in lymphocytes highly enriched with the double negative class. This population showed very high incidence of B lymphoid colony-forming cells compared to the unfractionated population (Lala & Jacobs, unpublished data). If this phenomenon represents a 'maturation arrest' of B cells *in vivo* the precise mechanism responsible for this effect remains unknown.

Since Ehrlich ascites tumour is a fluid tumour of unknown genealogy that can grow in many mouse strains, the relevance of the present findings to syngeneic solid tumours may be questioned. However, they are not unique to the present tumour; essentially identical results have also been obtained in this laboratory with the strain specific, syngeneic TA-3(St) mammary carcinoma line grown subcutaneously in strain A mice (Lala & Kaizer, 1977). Russell *et al.* (1976) detected a variable but significant number of lymphocytes which lacked detectable surface Ig or brain associated thymus antigen amongst inflammatory cells isolated from progressing as well as regressing murine Moloney sarcomas. In a preliminary study on a regressing subcutaneous transplant of Ehrlich ascites cells in CBA/H-T_6 mice (Lala & Kaizer, unpublished) we found an equally high incidence of double negative small lymphocytes within the transplant. This phenomenon, although immunological in nature, may not be tumour specific; sheep erythrocyte-induced peritoneal exudates in mice, although extremely low in lymphocyte content, also showed a high proportion of double negative small lymphocytes (Lala & Kaizer, unpublished). Further studies are underway to examine the functional potentials of double negative cells isolated from tumours as well as host spleens and the possible relevance of present findings to spontaneous murine tumours.

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