More Than Just Vanilla

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The year was 1969 and the immunological world mirrored the political world in its turmoil. Two camps of immunologists squared off: those who believed the thymus is a largely vestigial organ full of dying cells and those who believed this gland serves a crucial role, defined by the production of either hormones or cells, in the development and maintenance of the immune system (reviewed in Ref. 1). Those in the first camp could cite the apparently asymptomatic but dramatic postpubescent shrinking of the thymus in normal individuals and unambiguous data showing that adult thymectomy in mice minimally impacts spleen cell numbers and lifelong immunocompetence. Those in the "thymus is important" faction pointed to the findings that neonatally thymectomized mice have substantial immunological deficits as adults (2, 3) and that the effects of adult thymectomy were perhaps offset by the long lifespan of lymphocytes. This notion was reinforced by clinical observations in humans and by tracking immune function in chickens following ablation of the thymus or the bursa of Fabricius in late embryonic chicks, which suggested that two distinct lines of lymphocytes are dependent on two separate central lymphoid organs (reviewed in Ref. 4). Work conducted independently by the Miller and Mitchison groups clearly indicated that the generation of potent hapten-specific IgG Ab responses in mice required the coordinated contributions of spleen cells primed to carrier and those primed to hapten (5, 6). The function of the former cells was attributed to "antigen-specific handling," while the latter were presumed to be the Ab-secreting cells. Could these two functions elicited by priming with hapten and with carrier be performed by separate lineages of cells derived from distinct central lymphoid organs (the thymus on the one hand and the bursa or bone marrow on the other)? This fundamental question remained unanswerable as long as splenocytes were comprised of morphologically identical populations of lymphocytes. A marker to distinguish (and eventually separate) these two functionally discrete cell compartments was desperately needed. Proving once again that good things can come in small packages, that tall order was beautifully filled by a one figure, one table, three-column-long, single author paper by Martin Raff first describing Thy-1 (the theta isoantigen) as a marker of mouse T cells (7).

While working in Mitchison's division, Raff set out to test whether an antiserum recently generated by Reif and Allen in AKR mice injected with MHC-identical C3H thymocytes (8) could recognize a marker expressed by thymus-dependent lymphocytes. This antiserum appeared to identify an Ag on thymocytes from the most commonly used strains of laboratory mice. In line with the contemporaneous practice of using Greek letters to name serologically defined Ags, this marker was named θC3H (in honor of the immunizing cell type) and its allelic counterpart was named θAKR. Raff used the newly described ^{51}Cr-release assay to quantify cytolysis of CBA thymocytes, lymph node cells, and splenocytes in the presence of anti-θC3H antiserum plus hamster or guinea pig complement (7). By this simple but elegant assay, most thymocytes, over half of lymph node cells, and one-quarter to one-third of splenocytes were specifically recognized by this reagent. The distinct plateaus of cytolysis revealed that a discrete subset of θC3H$^+$ lymphocytes populates these primary and secondary lymphoid organs to a defined level. The thymus dependence of this subpopulation of lymphocytes was tested in specific pathogen-free mice treated with anti-lymphocyte serum, a reagent previously determined to deplete thymus-derived cells from the lymphoid periphery (9). Chronic treatment with anti-lymphocyte serum reduced the percentage of lymph node cells and splenocytes recognized by anti-θC3H antiserum by 80–90%. Thus, Raff had demonstrated in short order that the θ Ag serves as a marker for thymus-derived lymphocytes in the mouse. Schlesinger published similar findings at nearly the same time (10).

Less than four months (including two major holidays) later, Raff coauthored a paper demonstrating that surface Ig can be detected by autoradiography and immunofluorescence on a subset of peripheral lymphocytes (11) and that the surface Ig-positive and θ-positive lymphocytes represented nonoverlapping subsets (12). The longed for markers of bone marrow-derived and thymus-derived lymphocytes now had been realized. Raff also crafted the last piece of the puzzle by performing a reprise of the earlier Mitchison experiment adoptively transferring spleen cells from carrier-primed mice into lightly irradiated recipients that were then primed with hapten-conjugated carrier and assayed for hapten-specific serum Abs 10 days later. Treatment of carrier-primed splenocytes with anti-θ plus complement before transfer greatly diminished their ability to augment the recipient's Ab response (13). Thus, the surface marker for thymus-derived lymphocytes also identifies functionally distinct cells, tagging lymphocytes that help Ig-producing cells do their job. The era of "thymus-marrow synergism" (13) was born. Not all immunologists were immediate fans of this work, and one noted scholar disparaged the notion of functionally and phenotypically separate populations of lymphocytes, saying at the time that " 'b' and 't' are the first and last letters of 'bull- - - -.' " This immunologist will of course remain nameless.

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The notion that Abs can recognize antigenic markers of discrete cell lineages performing distinct functions is so fundamental to the way that immunologists design experiments now that it is difficult to imagine a time when this was not so. The Ab reagents have improved, moving from complex antisera to mAbs, some of the first of which were specific for Thy-1.1 and Thy-1.2, the current nomenclature for θAKR and θC3H, respectively. The cytolysis assay largely has been replaced by flow cytometry and magnetic bead separation, and Abs are still used routinely as a means to enrich and track defined subpopulations of cells. It is even more gratifying that this seminal discovery was presented in such a succinct and simple paper, with none of the grandstanding and self-aggrandizement we have come to expect in papers published today. Perhaps even more astounding, despite mentoring Raff, Mitchison declined authorship on this work, preferring instead to see the spotlight on this young scientist.

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Theta Isoantigen as a Marker of Thymus-derived Lymphocytes in Mice

THERE is an obvious need for a marker that will differentiate one type of lymphocyte from another. The need has become urgent in view of recent evidence suggesting that there are at least two populations of lymphocytes, one thymus-derived and one bone marrow-derived, which participate in different ways in the immune response[1]. The theta (θ) isoantigen (θ is determined by a single locus with two alleles: θAKR found in AKR and RF mice and θC_3H present in most other inbred strains of mice tested), described by Reif and Allen[2,3], which is found chiefly in thymus lymphocytes and brain, and to a lesser extent in peripheral lymphocytes in mice, seemed a possible antigenic marker of thymus-derived lymphocytes. To establish that θ is such a marker, it is necessary to demonstrate that there is a discrete population of peripheral lymphocytes which carry the antigen and that these cells are thymus-dependent.

Anti-θC_3H was prepared by injecting CBA thymocytes into AKR mice[3]. The cytotoxic activity of the antiserum was completely absorbed by adult CBA brain, confirming the specificity of the antiserum. ^{51}Chromium cytotoxic testing, as described by Wigzell[4] and modified by Bomford *et al.*[5], was used to detect θ on the surface of lymphocytes. Lymphocyte suspensions free of red blood cells were prepared[5] from adult CBA mice. The cells were labelled with $Na_2{}^{51}CrO_4$ and added to serially diluted anti-θ or normal AKR serum (NMS). After incubating for 30 min at 37° C, the cells were washed to remove the excess antiserum and resuspended in hamster or guinea-pig complement. A detergent ('Triton') was added to another set of tubes to kill all the cells and release their chromium. After a further 30 min incubation the reaction was stopped with cold saline. The tubes were centrifuged, and the supernatants decanted and counted in a well-type scintillation counter.

Fig. 1 shows the results of a representative experiment. The plateaux of cytotoxicity seen with high concentrations of anti-θ suggest that there is a discrete population of θ-bearing lymphocytes. In the case of the thymus, this consists of 97–100 per cent of the cells as judged by trypan blue dye exclusion cytotoxic testing[6]. This agrees with the findings of Wigzell[4] and Sanderson[7] that 70–80 per cent ^{51}Cr release from lymphoid cells corresponds to 100 per cent immune lysis. By extrapolation from ^{51}Cr cytotoxic testing and more directly from dye exclusion cytotoxic testing, approximately 70–80 per cent of lymph node lymphocytes and 30–50 per cent of splenic lymphocytes appear to carry the θ antigen in CBA mice.

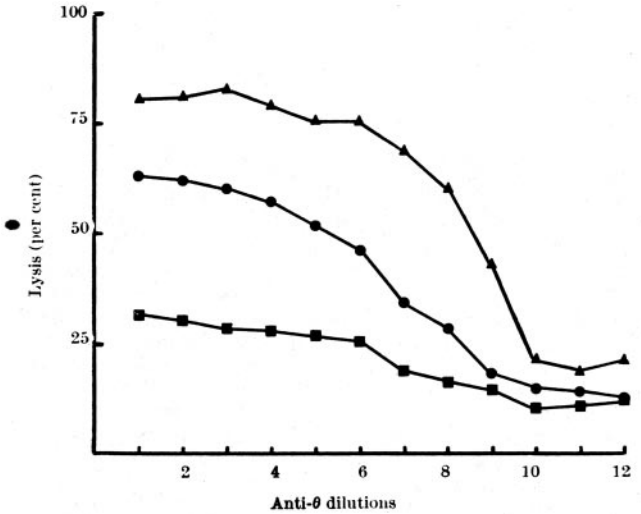

Fig. 1. Cytotoxic activity of anti-θC_3H on CBA thymocytes (▲), lymph node (●) and spleen lymphocytes (■). Tubes 1–12 contained doubling dilutions of anti-θ, beginning with 1 : 1 for lymph node and spleen and 1 : 10 for thymocytes. Per cent lysis = counts released with anti-θ/counts released with 'Triton'. Hamster complement, absorbed with mouse liver, spleen and thymus and diluted 1 : 7, was used with thymocytes. Guinea-pig complement, absorbed with mouse RBC and diluted 1 : 5, was used with lymph node and spleen.

To determine if this population of θ-bearing lymphocytes is thymus-dependent the θ content of lymph nodes and spleen lymphocytes in normal mice was compared with that of mice depleted of thymus-derived lymphocytes by chronic treatment with anti-lymphocyte serum (ALS)[8,9]. To minimize the effects of infection, CBA mice reared in a specific pathogen-free environment (SPF) were used.

One group of mice received ALS prepared according to the method of Levey and Medawar[10] beginning on the day of birth and given twice weekly until they were killed at 6–8 weeks old. Another group of mice received normal rabbit serum (NRS) according to the same schedule and some mice were left untreated.

Cytotoxic testing was carried out as before except the cells were incubated with anti-θ or NMS diluted 1 : 4 and hamster complement was used with all cell types. All tests were done in quadruplicate and the average number of counts released per tube with anti-θ, NMS, and 'Triton' was calculated and a cytotoxic index determined for each cell type by the following formula:

$$\frac{\text{Counts released (CR) with anti-}\theta - \text{CR with NMS}}{\text{CR with 'Triton'} - \text{CR with NMS}} \times 100$$

The results of three separate experiments are outlined

in Table 1. There was an 80–90 per cent reduction in the number of θ-bearing cells in the lymph nodes and spleens of ALS-treated mice when compared with the NRS-treated and untreated controls. There was no significant difference in the θ content of the thymuses in the different groups. Giemsa and methyl green pyronine stained smears of the various cell suspensions showed that in all cases at least 90 per cent of the cells were lymphocytes, mostly of the small variety. All experimental animals were autopsied and the only significant finding was a marked depletion of small lymphocytes in the thymus-dependent areas[11] of the spleen and lymph nodes in the

Table 1. CYTOTOXIC INDICES* (PER CENT) FOR THYMOCYTES, LYMPH NODE AND SPLEEN LYMPHOCYTES FROM CBA MICE TREATED WITH ANTI-LYMPHOCYTIC SERUM (ALS), NORMAL RABBIT SERUM (NRS) OR UNTREATED

	Mice	Thymus	Lymph node†	Spleen
Experiment 1	NRS-treated	89·8	51·4	16·4
	ALS-treated	80·8	7·1	1·7
	Per cent reduction‡	9·9	87	90
Experiment 2	Untreated	84·4	59·4	26·8
	NRS-treated	84	52·4	25·4
	ALS-treated	81·2	10·5	2·5
	Per cent reduction‡	3·35	80	90
Experiment 3	Untreated	70·4	53·3	
	NRS-treated	67·8	50·5	
	ALS-treated	76·3	6·1	
	Per cent reduction‡	—	88	

* Cytotoxic index =

$$\left[\frac{\text{Counts released (CR) with anti-}\theta\ (1:7) - \text{CR with NMS } (1:7)}{\text{CR with 'Triton'} - \text{CR with NMS } (1:7)}\right] \times 100$$

† Pooled mesenteric, inguinal, axillary, brachial, deep and superficial cervical.

‡ $\text{Per cent reduction} = \left(\frac{\text{NRS cytotoxic index} - \text{ALS cytotoxic index}}{\text{NRS cytotoxic index}}\right) \times 100$

ALS-treated mice. Both ALS-treated and NRS-treated mice showed prominent lymph follicles in lymph nodes and spleen with moderate numbers of plasma cells and lymphoblasts.

Thus, in CBA mice, by cytotoxic testing, 70–80 per cent of lymph node lymphocytes and 30–50 per cent of splenic lymphocytes appear to have the θ antigen on their surface. This population of theta-bearing lymphocytes in lymph node and spleen is strikingly reduced in mice which have been depleted of thymus-derived lymphocytes by chronic treatment with ALS. Preliminary experiments with newborn thymectomized mice, adult thymectomized lethally irradiated and foetal liver reconstituted mice, and mice with congenital absence of the thymus[12], have demonstrated a similar reduction of the θ content in lymph nodes and spleen[5]. This suggests that there is a discrete population of lymphocytes in the peripheral lymphoid tissues which carries the θ antigen and that these lymphocytes are thymus-dependent. θ therefore appears to be a useful marker for thymus-derived lymphocytes.

Since first submitting this report, Schlesinger and Yron[13] have reported a fall in the θ content in the lymph nodes of ALS-treated mice. Using dye exclusion cytotoxic testing, they were unable to detect θ-bearing cells in the spleen and did not estimate the relative number of θ-positive cells in the lymph nodes.

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