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Differentiation of functionally active mouse T lymphocytes from functionally inactive bone marrow precursors

I. KINETICS OF RECOVERY OF T-CELL FUNCTION IN LETHALLY IRRADIATED BONE MARROW RECONSTITUTED THYMECTOMIZED AND NON-THYMECTOMIZED MICE

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Summary. An investigation has been made of the development of various T cell functions in lethally irradiated mice reconstituted with anti-θ treated spleen or bone marrow cells. Evidence is presented to show that both organs contain a post-thymic precursor pool able to regenerate by 15 days limited T cell responses in thymectomized recipients. A prethymic pool also exists in each organ able to regenerate, at a later date, first a suppressor T cell population and probably later, mature functional T cells involved in helper functions and cell mediated lympholysis. The spleen is apparently a better source of precursors of the suppressor cells than bone marrow, while a poorer source of precursors of the other T cell functions. All T cell functions investigated apparently first appear in large cells which undergo a reversion to small cells without necessarily maturing to their full potential reactivity. By following the kinetics of appearance of T cell functions, and the physical parameters of the cells with which these functions are associated, it is shown that PHA responding and Con A responding cells, cytotoxic T cell progenitors, helper T cells for antibody production and helper T cells for cytotoxicity induction can all at some stage of differentiation be separated from one another.

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The authors regret that no reprints of this article will be available.

INTRODUCTION

Immune responses are classically defined as being of antibody or cell-mediated type, the two in turn being mediated by different classes of lymphocytes, B and T cells respectively. In recent years, considerable evidence has come to light indicating that each lymphocyte class is probably represented by a number of subpopulations which may be defined in terms of specific cell surface markers (Greaves, Owen & Raff, 1973). To date the field is at a relatively early stage in assigning independent functional properties to these subpopulations, or indeed in answering whether this will ultimately prove possible.

Early work by Cantor, Simpson, Sato, Fathman & Herzenberg (1975) suggested that the presence of varying concentrations of theta (θ) antigen on T lymphocytes could be used to distinguish a 'dull staining' T cell, sensitive to small doses of anti-

lymphocyte serum *in vivo*, which synergized (in the generation of cytotoxic cells) with a brightly staining T cell (long-lived and therefore not affected by adult thymectomy). Subsequently, using the Ly phenotype of T cells as a marker for subpopulations, Cantor & Boyse (1975) reported that Ly-1$^+$ cells represent a helper cell population for T-dependent antibody formation, and for generation of cytotoxic responses, while Ly2$^+$3$^+$ cells represent the progenitors of cytoxic T lymphocytes (CTL) to allogeneic cells themselves. By contrast, Shiku, Takahashi, Bean, Old & Oettgen (1976) reported that CTL for syngeneic tumor cells have the Ly123$^+$ phenotype. Feldman, Beverley, Dunkley & Kontianen (1975), and independently Cantor, Shen & Boyse (1975) have shown that a subpopulation of suppressor T cells also have an Ly1$^-$2$^+$3$^+$ phenotype. Rather more recently, a new I region locus (I–J) has been shown to be responsible for controlling cell surface determinants unique to these suppressor T lymphocytes (Ia–4) (Murphy, Herzenberg, Okumura, Herzenberg & McDevitt, 1976). In addition, Frelinger, Niederhuber & Shreffler (1976) have suggested that T cells responding to concanavalin A (con A) in serum free culture medium are likewise Ia positive cells (the locus for the determinant again mapping in I–J).

Stout & Herzenberg (1975) have similarly investigated the ability of Fc$^+$ receptor and Fc$^-$ receptor T cells to respond to various T cell mitogens and concluded that only Fc$^+$ receptor cells were responsive to con A.

An alternative approach to this problem of lymphocyte heterogeneity, which has already proven of use in analysing B lymphocyte subpopulations (Gorczynski, 1977a) is to investigate the kinetics of appearance of reactivity (in various T cell functional assays) from supposedly nonfunctional stem cells, and to analyse the physical properties of both the progenitors and effectors in these functional assays. In terms of size (Gorczynski & Rittenberg, 1974; Miller, Gorczynski, Lafleur, MacDonald & Phillips, 1975) and density (Shortman, 1974), considerable data are already available on investigations of mature T lymphocytes responding to mitogens and alloantigens (in proliferative and/or cytotoxic assays). By these criteria, moreover, there is reason to believe that mature T cells responding to phytohaemagglutinin (PHA) can be physically separated from those responding to con A (Gorczynski, 1976a), which is in keeping with the serological findings above (Fc$^+$/Fc$^-$ receptor bearing cells) and elsewhere (e.g., that low θ, high H-2 cells seem to be preferentially those cells responding to PHA and vice-versa (Shlesinger, Israël & Gery, 1976).

It is of interest not merely to ask whether mature T cell functional activities reside in biologically and/or physically separable cell populations, but also to question whether such functional heterogeneity in mature T cells can be correlated with some heterogeneity in bone marrow (prethymic precursors), or whether such heterogeneity develops within the thymus at a later stage of differentiation. In a recent publication, Huber, Cantor, Shen & Boyse (1976) have indicated that Ly1$^+$ or Ly23$^+$ T-cells adoptively transferred to T-depleted mice render (over the next 6 months) these animals capable of showing immune reactivity associated with their subpopulations only. Furthermore, the Ly phenotype of the adaptively transferred animal did not change in this time, probably ruling out the possibility that some type of precursor-progeny relationship exists normally between these cell types. We have studied in detail the kinetics of appearance of various T cell functions in lethally irradiated mice reconstituted with different size cell populations from the bone marrow (or spleen), and have tried to correlate these findings with the limited T cell differentiation/maturation which we have been able to induce *in vitro*. The data reported below and in the following manuscript give evidence for a prethymic and post-thymic pool of theta negative cells in both mature mouse bone marrow and spleen, though as yet we have little evidence for physical differences in the precursors of various T cell functions.

MATERIALS AND METHODS

Mice

Female C3H/He and AKR/J and male C3H/He and BALB/c mice were purchased from the Jackson Laboratories, Bar Harbour, Maine. (BALB/c × AKR/J)F_1 and (C3H/He × AKR/J)F_1 mice were bred at the Ontario Cancer Institute. All mice were kept 5 to a cage and fed food and water *ad libitum*. Mice were routinely used at 7–9 weeks of age.

Irradiation

Mice were irradiated with 1000 rad ionizing radiation from a ^{137}Cs source at a dose rate of 100 rad/min.

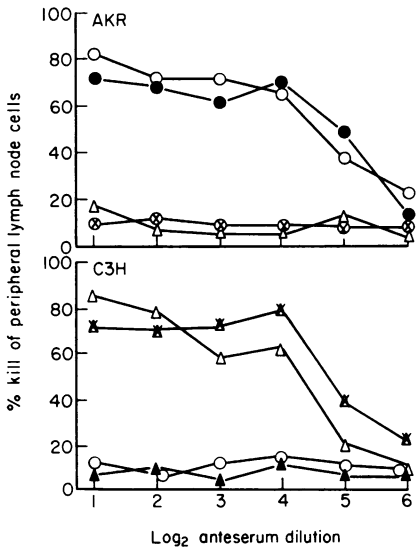

Figure 1. Trypan blue cytotoxicity tests on AKR anti-θ_{C3H} (△) and C3H anti-θ_{AKR} (○) antisera. 1×10^6 peripheral lymph node cells of either AKR/J (upper panel) or C3H/He (lower panel) were treated with various dilutions of unabsorbed sera (open symbol) or sera absorbed with C3H/He brain (closed symbols) or AKR/J brain (crosses within circles or triangles). After 60 min at 4°, the cells were washed and incubated for a further 45 min at 37° with rabbit complement (see Materials and Methods). Following this the cells were washed, stored on ice, and viability assessed by trypan blue dye exclusion. Concurrent controls showed the cytotoxicity of the rabbit complement in all cases was less than 5%. Per cent cytotoxicity was calculated as:

$$100 \times 1 - \left[\frac{\text{(viable cells/ml treated)}}{\text{(viable cells/ml untreated)}}\right].$$

Such mice were reconstituted with bone marrow cells within 2 h of irradiation, all injections being given intravenously (tail vein) in 0·5 ml phosphate buffered saline (PBS). Where cells were to be irradiated, the dose rate was 1500 rad and the cells were chilled on ice throughout.

Cell preparation

Cell suspensions of spleen, peripheral lymph node (axillary and inguinal only) and thymus were prepared as before by pressing chopped tissue through a stainless steel screen. Bone marrow suspensions were made by squirting phosphate buffered saline (PBS) through the femur and gently aspirating the plug of cells with a 10 ml pipette. Peritoneal exudate cells were prepared as described previously (Gorczynski, 1976b).

Antigens and Mitogens

Sheep erythrocytes (SRBC) were obtained from Woodlands Farm, Guelph, Ontario. Cultures were routinely immunized with 1×10^6 SRBC or 1 ng/ml DNP-lys-Ficoll (prepared following the technique of Sharon, McMaster, Kask, Owens & Paul (1975)).

Phytohaemagglutinin (PHA) and concanavalin A (con A) were both purchased from Difco Labs, U.S.A. Their final concentration in culture was 1% or 3 μg/ml respectively.

Antisera

AKR anti-θ_{C3H} and C3H anti-θ_{AKR} antisera were prepared by inoculating the relevant recipients with $1{\cdot}5 \times 10^7$ donor thymocytes weekly for 6 consecutive weeks and bleeding 7 days after the final injection. Assayed against C3H or AKR peripheral lymph node the cytotoxic indices of the relevant sera were as shown in Fig. 1. By such criteria (specific kill of 60–70% of lymph node cells, completely abolished by absorption with the donor strain brain tissue), we concluded that these sera were indeed strain specific. Further functional tests (as described elsewhere, Gorczynski, 1976b) established the specificity of killing (for con A responsive T cells and not lipopolysaccharide (LPS)-responsive B cells). Routinely, these sera have been used at a 1/10 dilution.

Rabbit anti-mouse brain theta associated antigen (anti-Brθ) was prepared and tested as reported earlier (Golub, 1971; Gorczynski, 1974). This serum was routinely used at a 1/30 dilution. All serum treatments were performed in a 2-step fashion (60 min at 4° with serum followed by 45 min at 37° with rabbit complement) as described before.

Velocity sedimentation

Unit gravity velocity sedimentation, which separates cells predominantly on the basis of their size, was performed as described elsewhere (Miller & Phillips, 1969). A sterile glass sedimentation chamber (model SP-120, Jolin's Glass, Toronto) was used and sedimentation was for 4 h at 4°.

Cultures

All cells were cultured in α-MEM (Flow Laboratories, Rockville, Md) containing 2-mercaptoethanol (2ME) (5×10^{-5} M) and 10% fetal calf serum (αF_{10}). Cultures for antibody forming cell (PFC) induction were set up in sterile glass tubes (12 × 75 mm), $3{\cdot}0 \times 10^6$ cells being cultured in 1·5 ml

medium. For cytotoxic T cell induction (CTL), 2·5 to 7·5 × 10^5 cells were cultured with 2·5 × 10^5 irradiated spleen cell targets in 1 ml medium in sterile glass tubes (10 × 75 mm). All cultures routinely also contained 1 × 10^5 irradiated anti-Brθ treated peritoneal cells to saturate for any requirement for accessory cells (Gorczynski, 1976b). All cells were cultured for 5 days at 37° in a humidified atmosphere with 10% CO_2.

Antibody forming cell assays

The PFC assay used was that developed by Cunningham & Szenberg (1968).

Cytotoxicity assays

The ^{51}Cr assay used throughout has been described in detail elsewhere (Gorczynski, 1976b). Cytotoxicity was measured using 1 × 10^4 ^{51}Cr labelled P815 tumour cells as targets with varying numbers of effector cells. Per cent specific cytotoxicity was calculated as:

$$100 \times \frac{\text{(c.p.m. experimental} - \text{c.p.m. spontaneous)}}{\text{(c.p.m. detergent} - \text{c.p.m. spontaneous)}}.$$

We routinely found that within a given experiment, plots of cytotoxicity versus log effector cell number for various groups of cultures consisted of a series of parallel straight lines. Thus the relative cytotoxicity of each culture group was compared by examining the cytotoxicity derived from a constant percentage (generally 5%) of the recovered culture.

Mitogen and mixed leucocyte stimulation cultures

This technique is described in detail elsewhere (Gorczynski & Rittenberg, 1974). 2 × 10^5 responder cells were added (in 100 μl) per culture and 2 × 10^5 irradiated anti-Brθ treated syngeneic spleen cells were also added to saturate for any requirement for accessory cells. (In our hands, such irradiated cells alone never generated a significant proliferative response to mitogen or allogeneic cells.) Mitogens or 1 × 10^5 irradiated F_1 hybrid stimulator cells [(BALB/c × AKR)F_1 mice] were added in 20 μl and the cultures were incubated for 72 h. After this period of time, all cultures were washed and pulsed for 8 h with 1 μCi of [^{3}H]thymidine in 100 μl αF_{10}. The cells were then washed with 1 × 10^6 fresh normal spleen cells as carrier, precipitated with 10% trichloracetic acid (TCA), harvested on Whatman glass fibre filter papers and counted in a well type

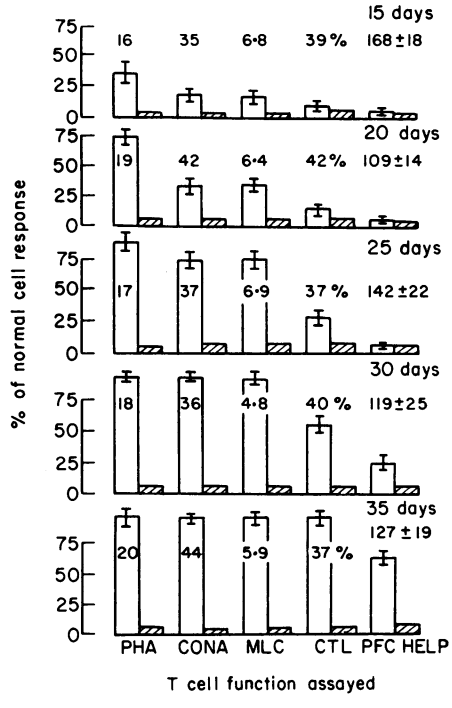

Figure 2. Recovery of different T cell functions in irradiated bone marrow reconstituted mice. These data are explained in more detail in the text and Materials and Methods. The bars represent the reactivity (relative to normal spleen cells) of pooled recipient spleen cells in the various assays before (open) or after (hatched) treatment with C3H anti-θ AKR antiserum. The responses of normal spleen cells are the figures shown above each bar, i.e. stimulation indices for the proliferation assays and per cent cytotoxicity or PFC/culture for the other assays. On all days of assay, the response of 2 × 10^6 anti-θ treated normal spleen cells was less than 10 ± 4 PFC/culture. Helper T cells for SRBC-PFC were determined by the addition of 1 × 10^6 spleen cells to 2 × 10^6 anti-θ treated normal cells. Data for CTL are shown for cultures containing initially 5 × 10^5 responder spleen cells.

scintillation counter. The stimulation index was calculated as:

$$\frac{\text{(c.p.m. in presence of stimulus)}}{\text{(c.p.m. in presence of } \alpha F_{10} \text{ only)}}.$$

A value of 1·0 indicated no significant response. For all assays used, the standard errors of the experiments subsequently reported were in general less than ± 12·5 %.

RESULTS

Kinetics of recovery of different T cell functions from unfractionated bone marrow cells

In a preliminary series of experiments, it was found that irradiated mice given no bone marrow inoculum had detectable T-responder cells (PHA, con A and MLR responsive cells, as well as precursors of cytotoxic lymphocytes) at least until days 12–14, at which time the mice died from the lethal irradiation. Accordingly, it became imperative to ensure that any T responder cells assayed were donor-derived in all assays used. We have thus resorted in all experiments reported herein, to the use of lethally irradiated (C3H/He × AKR/J)F_1 mice as recipients, and anti-Brθ treated AKR/J cells as donor bone marrow cells. Anti-θ treatment of the bone marrow was deemed essential in view of auxiliary information that bone marrow contained a small percentage of theta-positive cells which were able to respond in the proliferative assays used (mitogen and MLC cultures). When the recipients were killed and their spleens assayed for regenerating T cells, the cells were first pretreated with AKR anti-θ_{C3H} antiserum to remove any surviving host T lymphocytes.

The data of Fig. 2 indicate the regeneration characteristics of 5 independent T cell functions assayed at various times after inoculation of $1{\cdot}5 \times 10^7$ (anti-Brθ treated) bone marrow cells into irradiated mice. At each day of assay, 3 spleens were pooled for use as the test regenerating spleen cell pool, and spleens were similarly pooled from 3 normal AKR/J mice as control cells. All cells were treated with anti-θ_{C3H} and complement. One half of each cell pool was subsequently treated with anti-θ AKR antiserum and complement. Mitogen and mixed leucocyte proliferation assays were performed on an aliquot of each cell suspension as described in the Materials and Methods. Similarly, we assayed aliquots of the cell pools for their ability to generate cytotoxic T lymphocytes in response to stimulation with irradiated (BALB/c × AKR)F_1 spleen cells, and to provide helper T cells for the SRBC-PFC response of 2×10^6 anti-θ treated normal AKR/J spleen cells. The data of Fig. 2 represent arithmetic means (± standard error) of 3 cultures for each assay. Open bars show untreated cell suspensions, hatched bars show cells treated with C3H anti-θ AKR antiserum. This experiment is representative of many investigations (one of twelve).

There are several points of interest in these data. Firstly, it is apparent that at all times the measured T cell responses were donor cell derived (that is, sensitive to anti-θ_{AKR} antiserum). This has been observed (using the protocol above) routinely in this system and no further tests to document this fact are described in the remaining experiments reported. Secondly, we found that there was a definite time sequence for the appearance of reactivity in these assays. PHA responsiveness appeared first (by 15 days though not to any great extent by day 12—data not shown) followed rapidly by simultaneous responsiveness to con A and in MLC proliferation, and only much later by the ability to generate cytotoxic T lymphocytes or T helper cells (for SRBC-PFC). Finally, and perhaps even more strikingly, was the evidence that to a degree some regeneration of all responses (with the sole exception of help for PFC responses) took place by day 15. As shown, while small relative to the cytotoxicity seen from normal spleen cells, significant potential to develop cytotoxic T cells had also developed by day 15, though no appreciable further increase took place until much later times (day 25 here).

Analysis of precursors of T cells responding at days 15 and 30 in reconstituted mice

Two possible explanations, which are not mutually exclusive (see below), were considered for the apparent biphasic reconstitution of T cell responses seen. The first was that a more committed (to the T lymphocyte axis of differentiation/maturation) precursor was responsible for the early burst of T cell activity (day 15) while a more immature cell was predominantly responsible for the later activity (day 30 reconstituted mice). The second was that during the period from 15 to 30 days, a transient production of suppressor cells took place. This could, in turn, lead to 'false-negatives' in some of the assays (PFC and cytotoxicity) used.

In order to investigate whether different precursor populations were responsible for the activity measured in day 15 or day 30 reconstituted mice, we analysed the sedimentation characteristics of the bone marrow pool used to reconstitute the animals.

Bone marrow cells were pooled from 20 AKR/J donor mice and 4×10^8 of the cells were sedimented at 4° for 4 h from an initial starting band of 40 ml. Fractions corresponding to cells differing in sedimentation velocity by 1·0 mm/h were collected, the cells centrifuged (500 *g* for 5 min at 4°), resuspended

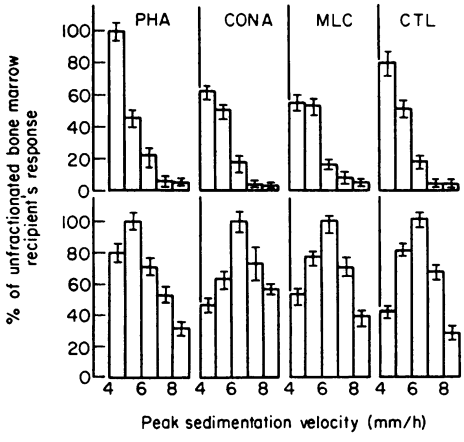

Figure 3. Sedimentation analysis of precursor bone marrow cells responsible for reconstitution of T cell dependent functions in irradiated hosts at 15 (upper panels) and 30 (lower panels) days after bone marrow inoculation. This experiment is described in greater detail in the text. All bars represent the responses of pooled spleen cells (at least 3 per group) from recipients given bone marrow populations of different sedimentation velocity relative to the responses seen with recipients of unfractionated bone marrow cells. The response of these latter recipients (as well as of fresh normal AKR/J spleen cells) at the 2 days of assay were as follows:

	Day 15		Day 30	
	Irradiated	Normal	Irradiated	Normal
PHA (stimulation index)	6·2	16	16	22
Con A (stimulation index)	9·6	42	33	39
MLC (stimulation index)	2·1	7·2	5·2	6·8
CTL(% cytotoxicity)	4·9	49	37	45

in αF_{10} and counted. All fractionated cells as well a 1×10^8 of an unfractionated control sample were then treated with anti-Brθ and rabbit complement, washed twice, and inoculated into 9 irradiated (C3H/He × AKR/J)F_1 per group. 15 days and 30 days after inoculation, a minimum of 3 donors per group were killed, the spleens within a group pooled, and the cells used to investigate the recovery of the potential to respond in mitogen and MLC assays, and in cytotoxicity assays. The data from a typical experiment (one of six of this type) are shown in Fig. 3.

The upper panels of this Figure demonstrate quite clearly that all day 15 T-cell responses were regenerated from a pool of bone marrow precursors sedimenting in the region 4 to 6 mm/h. There was, from this and other repeat experiments, a certain amount of evidence that the smaller cells of this pool may be preferentially committed to differentiation/maturation to PHA responder T cells. The data in the lower panels of this figure, obtained from 30 day reconstituted mice, were no less informative. It was apparent that the bone marrow precursors for T cells responding at this time were no longer to be found in a unique region of the gradient, but rather it seemed that all cell fractions reconstituted to a high degree, with the optimum regeneration being in the region 5–8 mm/h.

The great difference in the breadth of sedimentation profiles of biological precursor activity seen when functional T cell reconstitution was measured at day 15 or 30, could best be explained in terms of the fact that the longer the regeneration time the more other limiting factors (e.g., number of cell divisions initiated from a given precursor pool in individual mice, generation of suppressor cells of a specific/non-specific nature, etc.) may affect the ultimate measured activity. If other variables do have a significantly greater effect the longer the assay period, we should expect the assay to be less quantitative (for progenitor cells) with time elapsed between inoculation and testing. If this were so, the width of the biological activity profile would then not represent a true reflection of the relative biological activity potential (at time 0) in individual fractions.

Since we knew from earlier studies (Gorczynski, 1977b) and current (unpublished) studies with regenerating spleen cells that the functional assay systems used (72 h proliferation assays, 5 day CTL assay, etc.) were of a quantitative nature, we were able to investigate in a direct fashion the degree of quantitation we could apply to our analysis of the progenitor cells (of mature T cell function). Accordingly, groups of 9 irradiated (C3H/He × AKR/J)F_1 mice were inoculated with graded doses of anti-Brθ treated AKR/J bone marrow cells. After either 15 or 30 days, standard cultures were initiated (using spleen cells pooled from 3 recipients of each type) to measure mitogen responses to Con A (crosses in Fig. 4) or CTL generation in the presence of irradiated (BALB/c × AKR/J)F_1 cells (circles in

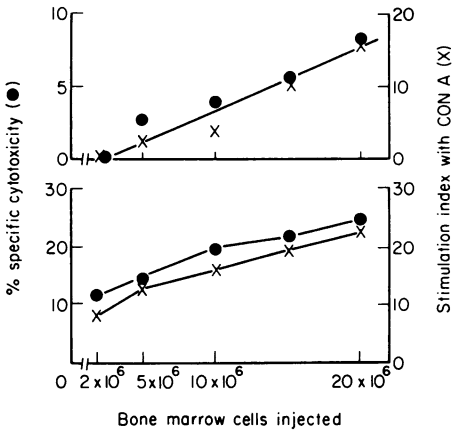

Figure 4. Dose–response curve for regeneration of CTL (●) or Con A responder T cells (×) in irradiated bone marrow recipient mice assayed at day 15 (upper panel) or day 30 (lower panel). These experiments are described in more detail in the text and in the Materials and Methods. All points represent the arithmetic mean of 3 cultures. At the two times of assay, fresh normal AKR/J spleen cells gave the following responses:

	Day 15 assay	Day 30 assay
Con A stimulation index	46 ± 3·7	42 ± 3·1
CTL (% specific cytotoxicity)	54 ± 2·2	46 ± 2·9

Fig. 4). These data (one of three experiments) are shown in Fig. 4.

It was apparent that the assay of precursor cells responsible for regeneration of CTL progenitors or con A responder T cells was linear with respect to the number of bone marrow cells in the donor innoculum when the assay for T function was made at day 15. These precursors sedimented in the region 4 to 6 mm/h (Fig. 3). In contrast, when the assay of T function was delayed till day 30 very little quantitation (with respect to the number of marrow cells injected) was possible (see lower panel of Fig. 4). This lack of quantification could be used to explain the broad biological activity profile seen in Fig. 3, panel b). We suggest that the major activity resided at 6 to 7 mm/h, and was in reality (at time 0) associated with a far sharper peak than suggested by assaying at day 30. The measured peak was broadened by 'tailing' into the larger and smaller cell regions of the gradient of progenitors which, by day 30 (of assay), had provided activity to a similar degree to the cells from 6 to 7 mm/h though they intitially contained far fewer progenitors than this pool of cells. Another possible explanation for the breadth of this profile at day 30, and the lack of quantification, could be that an even slower regeneration of T cell functions was still occurring from a yet more immature stem cell than that responsible for the majority of the day 30 activity. The profile of this precursor cell was being superimposed on that of the major activity pool we sought to measure. Arguing against this somewhat are our findings (unpublished) that the transition from the day 15 profile, even to a day 50 profile, seemed to be correlated only with the gradual change from 4 to 6 mm/h precursors to 5 to 8 mm/h precursors as in Fig. 3.

In a final attempt to characterize differences between the cells responsible for early and late reconstitution of T cell function in irradiated bone marrow reconstituted mice, we have investigated the thymus dependency of the development of such T cell function. Adult (7 week) (C3H/He × AKR/J)F_1 mice were thymectomized under ether anaesthesia and 2 weeks later were used as irradiated recipients of 10×10^6 anti-Brθ treated AKR/J whole bone marrow cells, or 5×10^6 anti-Brθ treated cells sedimenting in the region 4·0–6·0 or 6·0–8·0 mm/h. Sham thymectomized controls were similarly irradiated and bone marrow reconstituted. 15 days and 30 days following bone marrow inoculation, 3 mice of each group were killed, the spleens of each individual group pooled (as were 3 fresh normal AKR/J spleen cell preparations) and standard T-cell assays performed on the resultant cell preparations. The data for one experiment (of 3) of this type are shown in Table 1.

As reported already (see Figs 2–4), mice receiving unfractionated bone marrow cells or cells in the pool 4–6 mm/h showed evidence of reconstitution of T cell function by day 15, while cells from the pool sedimenting in the region 6–8 mm/h were not able to reconstitute at this time. Moreover, this pattern of reconstitution at day 15 was evident even in the spleen of thymectomized irradiated recipients. All recipients were analysed at killing for thymic remnants and any with evidence of incomplete thymus removal were not used for experimental analysis. Further ruling out the notion that incomplete thymus removal was responsible for this phenomena were the data from 30 day reconstituted animals which indicated, as classically reported, that

Table 1. Role of thymus in early and late regeneration of T cell function from purified bone marrow populations

	Reconstituting* cell population under test	Thymectomized† recipients		Sham-thymectomized† recipients	
		Per cent cytotoxicity	Stimulation index with Con A	Per cent cytotoxicity	Stimulation index with Con A
Day 15 assay	Whole bone marrow	7·6 ± 0·9	9·5 ± 1·7	9·5 ± 1·7	8·7 ± 1·3
	4–6 mm/h	6·8 ± 0·4	12 ± 1·9	7·2 ± 0·9	11 ± 1·8
	6–8 mm/h	0 ± 0	1·4 ± 0·1	0 ± 0	1·2 ± 0·1
	Normal spleen‡	56 ± 3·9	37 ± 4·2		
Day 30 assay	Whole bone marrow	4·5 ± 0·5	7·8 ± 1·9	46 ± 2·6	37 ± 2·2
	4–6 mm/h	4·1 ± 0·6	8·2 ± 1·3	19 ± 1·7	20 ± 1·8
	6–8 mm/h	0 ± 0	1·1 ± 0·1	52 ± 2·9	33 ± 2·6
	Normal spleen‡	47 ± 4·6	33 ± 3·9		

* 10×10^6 (whole bone marrow) or 5×10^6 of either of the velocity sedimented cell pools were used for reconstitution. Recipients were sacrificed on the 2 days of assay shown, 3 mice per group being used to prepare the relevant spleen cell pool for testing (subsequent columns).

† All (C3H/He × AKR/J)F_1 recipients were given 1000 rads prior to injection. All spleen cell pools were treated with AKR anti-θ C3H prior to test. Details of the cytotoxicity and mitogen assays are to be found in the Materials and Methods.

‡ The activity from pools of fresh normal AKR/J spleen cells at the days of assays shown.

these animals showed T cell deprivation *unlike* the sham thymectomized controls. While some reconstitution from the 4–6 mm/h pool was still evident at this time, it was not analogous to the reconstitution in sham thymectomized recipients of the same cell pool, and seemed even to be less than the degree of reconstitution seen at day 15 in these mice. Certainly the day 30 reconstitution seen from cells sedimenting in the region 6–8 mm/h was totally abolished in thymectomized mice.

These data argued strongly that not only were the sedimentation and titration characteristics of cells reconstituting at day 15 and day 30 quite different, but the thymus dependency of that reconstitution was also quite different. In fact early regeneration of T cells was due to a post-thymic (but anti-Brθ resistant) population of cells which seemed to give rise to relatively short lived T cells, while later reconstitution was from a prethymic (stem-cell?) pool. It seemed that regeneration from the latter was more likely to represent differentiation of stem cells into mature T cells while regeneration from the former more closely mimicked a maturation phase in T-cell development.

Sedimentation velocity of regenerating T cells at different times after bone marrow inoculation

The data in Figs 2 and 3 had already suggested that in gross kinetic terms we could demonstrate apparently separate populations of T cells recovering reactivity in different assays. An alternative interpretation of these data would be, however, that the same T cell was responsible for all functions assayed, but recovered the ability to provide these functions in discrete steps. In order to explore further the possibility that the various T cell functions developing in bone marrow reconstituted mice were associated with physically different cells, and were perhaps derived from physically separable progenitors, we investigated the sedimentation velocity of functionally active T cells in irradiated recipients of previously fractionated AKR/J bone marrow cells.

Bone marrow was obtained from 30 AKR/J donor mice. The cells were fractionated for 4 h at 4° and fractions corresponding to cells sedimenting in the ranges (4·0–6·0), (6·0–7·5) and (7·5–10) mm/h were collected. All cells were treated with anti-Brθ and rabbit complement, washed twice and then injected

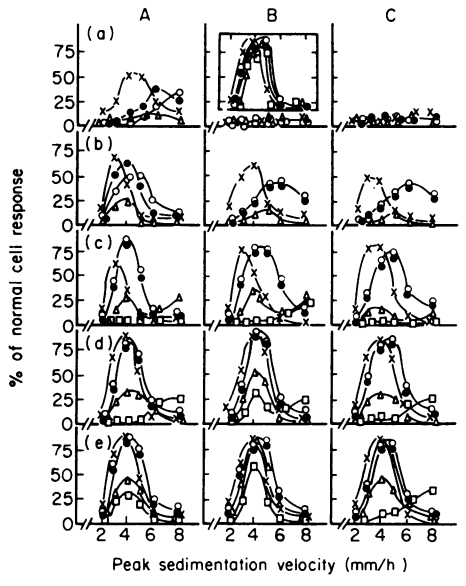

Figure 5. Sedimentation analysis of regenerating T cells in mice receiving fractionated bone marrow cells. These data are described in more detail in the text and Materials and Methods. Panels (a) to (e) represent data obtained at different days of sacrifice (15–35 as represented in Fig. 2). Columns A, B and C represent the data obtained using groups of mice receiving bone marrow cells with sedimentation velocity: (4–6), (6–7·5) and (7·5–10) mm/h respectively. All T cell functions, as in Fig. 2, are expressed relative to an unfractionated normal AKR/J spleen cell control sample assayed at the same time. The following functions were assayed: (×) PHA mitogen response; (○) MLC proliferation (in response to irradiated (BALB/c × AKR/J)F_1 cells); (●) Con A mitogen response; (△) CTL generation in culture; (□) Help for SRBC-antibody response (using anti-θ treated AKR/J spleen cells as a source of B lymphocytes). These data are only shown in panels (c) to (e) since prior to this, no reactivity was demonstrated in any fraction of columns A to C.

The insert to panel (a) column B is typical of data seen when analogous sedimentation analysis was performed on a fresh normal spleen cell pool.

The control data (with normal spleen cells) at each day of assay were as follows (at no time did anti-θ treated AKR/J spleen cells alone show a response to SRBC above 10 PFC per culture).

Day of assay	Stimulation indices			% cytotoxicity CTL	SRBC-PFC response
	Con A	PHA	MLC		
15	41 ± 2·7	17 ± 2·2	6·8 ± 0·9	51 ± 2·6	136 ± 19
20	33 ± 3·6	26 ± 2·8	4·7 ± 0·7	43 ± 3·9	109 ± 11
25	39 ± 2·9	22 ± 4·1	6·2 ± 0·8	56 ± 4·1	129 ± 22
30	40 ± 4·7	21 ± 2·7	5·9 ± 1·0	42 ± 2·7	158 ± 16
35	26 ± 2·8	19 ± 2·4	6·1 ± 1·2	47 ± 4·2	127 ± 17

into 20 recipients (C3H/He × AKR/J)F_1 mice per group. On each of days 15, 20, 25, 30 and 35 following bone marrow reconstitution, a minimum of 3 mice per group were used to prepare a spleen cell pool (as were 5 normal fresh AKR/J mice). 90% (or not greater than $2·0 \times 10^8$ cells) of each spleen cell pool was then itself fractionated by velocity sedimentation for 3 h at 4°, cell fractions sedimenting with differences in velocity of 1 mm/h collected, and the cells centrifuged, resuspended in αF_{10} and counted. T cell assays (as in Fig. 2) were performed on all fractions as well as on unfractionated control samples, the data being shown in Fig. 5 (1 out of 6 experiments).

There were many interesting features in this figure. With respect to all T functions analysed, it seemed that the first appearing cell was much larger than the cell with equivalent function in normal spleen, and that with time after reconstitution a gradual reversion to smaller cells took place. Thus a reversion from large responding cells to small responding cells was seen for Con A (●–●) and MLC (○–○) responses in column A (a, b, c) and columns B and C (b, c, d). Similarly, for generation of cytotoxic cells (CTL, △–△) the effect was evident in columns A (a, b), B (b, c, d) and C (b, c) though there was a biphasic distribution of CTL progenitors at day 25 (panel c), the cause of which is discussed further below. Helper cells for antibody production (□–□) similarly went through this large to small cell transition (columns B and C (c, d, e) and column A (d, e)).

A further point of interest was that at some stage of differentiation we could define unique subclasses of T cells involved in discrete responses. Thus, the already documented size differences between PHA and Con A responding cells in normal spleens (see insert panel a column B) was further dramatized at early times after bone marrow transfer (see ×–× in A (a, b) and B (b), C (b)). Moreover CTL progenitors (△–△) and helper cells (for antibody production (□–□)) were quite distinguishable from each other and other T cells (e.g., A (c, d), B (c, d) and C (c, d, e)). Evidence for a separation of Con A and MLC responding cells was seen only using cells from an alleged post-thymic pool at early times after marrow grafting (see ○–○ and ●–● in A (a, b)).

It was also apparent from the curves for CTL progenitors that these cells could apparently complete their size transition (e.g. in panel d columns A and B, all T cell activity resided in small cells)

without becoming functionally active (e.g. the rise in activity in such small cells from panels d to e). This phenomenon was also observed for helper T cells for antibody production where the size transition seemed complete by day 35 (panel e column B), but further increase in activity in the small cell region of the gradient was apparent up till day 50 (data not shown). Such a conclusion rested on the assumption that no activity measured in small cells was derived from an inactive larger cell, but the uniformity with which active large cell to active small cell transitions were observed with each assay suggested that this was not an unreasonable assumption. Finally, and in support of the notion that the peak sedimentation velocity of prethymic bone marrow precursors was indeed in a narrower sedimentation range (6–7 mm/h) than suggested in Fig. 3 (5–8 mm/h), it was apparent that in most cases the transition to size characteristics (and net activity) typical of normal spleen cells occurred rather earlier in column B than in column C or A (if one attempted to take into account in a qualitative fashion the contribution made in A by the unique class of post-thymic precursor cells).

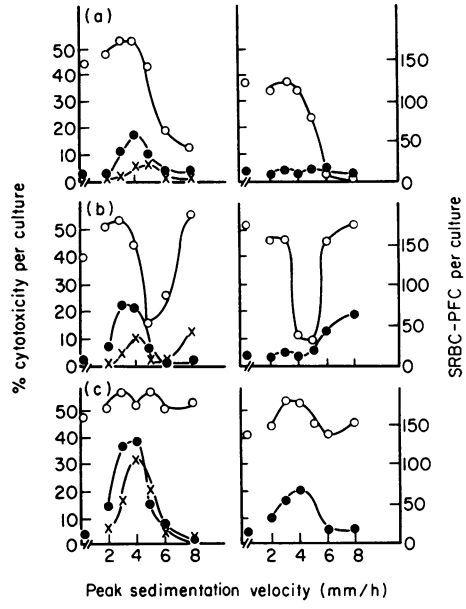

Figure 6. Early generation of suppressor cells in bone marrow reconstituted mice. These experiments are described in greater detail in the text. Panels a, b and c respectively indicate sedimentation of spleen cells from 20 day, 25 day and 30 day reconstituted mice. The left hand panels represent cytotoxic responses, the right hand panels SRBC-PFC cultures. Open circles represent cultures in which we tested for suppression of the control response obtained from normal spleen cells (to extreme left of each panel). Closed circles indicate cultures testing for helper cells. In the cytotoxic assays, this involved assaying for synergism with 1×10^5 normal AKR peripheral lymph node cells (cytotoxicity from these alone is shown to the left of these panels). In the PFC assays, this involved assaying for synergism with 2×10^6 anti-Brθ treated normal AKR spleen cells (control data with θ-treated cells alone are again to the left of each panel). The crosses indicate the cytotoxic response seen with the fractionated spleen cells themselves.

Complexity in sedimentation analysis introduced by differentiation of suppressor cells

Detailed analysis of the curves in Fig. 5 analysing the generation of precursors of CTL revealed that there was an apparent exception to the large cell—small cell transition referred to above. Namely at day 20 (panel b), all cytotoxic T cell progenitors seemed to exist in a pool of small cells while at a later date (panel c) there was evidence for large cell precursors of CTL. If this was not an exception to the aforementioned phenomena, then these data suggested that the potential of large cells (at day 20 of reconstitution) to develop into CTL was being masked by the presence of an inhibitor in the same region of the gradient. Similar reasoning could perhaps be applied to the long delay in the appearance of helper cells (for PFC formation). Accordingly, we reinvestigated the sedimentation profiles (only progenitors of CTL and helper cells involved in antibody production) analysed in Fig. 5 with the purpose of identifying any potential inhibitor cell pool.

Bone marrow cells were obtained from 20 AKR/J mice and sedimented for 4 h at 4° as before. Cells in the region 6–8 mm/h were pooled, treated with anti-Brθ and rabbit complement and inoculated into 15 irradiated (C3H/He × AKR/J)F_1 hybrid mice. At 20, 25 and 30 days post irradiation, spleen cells were pooled from a minimum of 3 mice per day, and a similar cell pool was prepared from 5 fresh normal spleen cells. $1{\cdot}0 \times 10^8$ of each pool of cells was then sedimented at 4° for 3 h, fractions corresponding to differences in size of 1·0 mm/h collected and the cells centrifuged and counted. For each cell pool, we then investigated 5 functions:

(i) Ability to generate CTL directly (from 5×10^5 cells).

(ii) Ability of 5×10^5 cells to amplify the CTL

responses from 1×10^5 AKR/J peripheral lymph node cells (help in CTL response).

(iii) Ability of $5{\cdot}0 \times 10^5$ cells to inhibit the CTL responses from 5×10^5 normal AKR/J spleen cells.

(iv) Ability of 1×10^6 cells to help the SRBC-PFC responses of 2×10^6 anti-Brθ treated normal AKR/J spleen cells.

(v) Ability of 5×10^5 cells to inhibit the SRBC/PFC response of $2{\cdot}5 \times 10^6$ normal AKR/J spleen cells.

The data from one of the 5 experiments of this type are shown in Fig. 6.

These data indicated the extreme complexity involved in investigating regenerating responses in bone marrow protected mice. It was apparent that at day 20, large cells existed in the spleen of these recipients which were capable of inhibiting the response of a normal AKR/J spleen cell preparation in both cytotoxic and PFC assays. Thus, we cannot yet suggest whether the profiles generated for helper cells and cytotoxic cells at these times were correct, since the inhibitor could be masking activity in fractions which simultaneously contain inhibitors. It was quite possible, and we felt likely (in view of the uniformity of such observations for other T cell functions) that for cytotoxic cell progenitors too, the first active cell (day 20?) was a predominantly large cell which then underwent a gradual shift to become a smaller cell. It was also possible that larger helper cells (for SRBC-PFC) existed already at day 20, though we were unable to measure them directly until day 25 by which time the inhibitor cell had also become a small cell (panel b). An interesting feature of this figure was the indication that helper cells for cytotoxic responses (●–●), which were also C3H anti-θ AKR sensitive (data not shown here) seemed to be unlike those required as helper T cells for SRBC-PFC function. In fact, they were also unlike progenitors of cytotoxic cells and more closely resembled (in terms of sedimentation characteristics) MLC and Con A responding cells, a point further documented in subsequent analysis (unpublished data and Figs 3 and 5).

It should be pointed out here that at no time did we observe any inhibition from any cell fraction of normal spleen cells at the doses used. Moreover,

Table 2. Effect of thymectomy on development of suppressor cells in irradiated bone marrow reconstituted mice

Added suppressor cells* (day 20/25 reconstituted mice)			Cytotoxic† response per culture	SRBC-PFC‡ per culture
None			41 ± 3·6	168 ± 27
Sham thymectomized	Day 20	$1{\cdot}0 \times 10^5$	19 ± 3·6	160 ± 26
		$2{\cdot}5 \times 10^5$	7·8 ± 0·9	107 ± 14
		$5{\cdot}0 \times 10^5$	1·6 ± 0·2	22 ± 6
	Day 25	$1{\cdot}0 \times 10^5$	26 ± 2·1	142 ± 38
		$2{\cdot}5 \times 10^5$	13 ± 1·6	88 ± 17
		$5{\cdot}0 \times 10^5$	6·7 ± 0·5	17 ± 5
Thymectomized	Day 20	$1{\cdot}0 \times 10^5$	38 ± 4·7	141 ± 28
		$2{\cdot}5 \times 10^5$	34 ± 4·2	119 ± 19
		$5{\cdot}0 \times 10^5$	33 ± 2·9	122 ± 16
	Day 25	$1{\cdot}0 \times 10^5$	39 ± 2·8	118 ± 21
		$2{\cdot}5 \times 10^5$	41 ± 5·6	131 ± 11
		$5{\cdot}0 \times 10^5$	32 ± 2·8	120 ± 19

* Suppressor cells were obtained from the large (6 to 9 mm/h) or medium-sized (4·5 to 6·5 mm/h) region of a sedimentation gradient of 3×10^8 cells from day 20 or day 25 reconstituted mice.

† All cultures received 5×10^5 normal AKR/J and $2{\cdot}5 \times 10^5$ irradiated (BALB/c × AKR/J) spleen cells with graded doses of inhibitors as shown.

‡ All cultures received 1×10^6 SRBC and $2{\cdot}5 \times 10^6$ normal AKR/J spleen cell along with inhibitors as shown.

regenerating spleen cells themselves seemed to develop the inhibitors only during the brief period from 18 to 28 days, the active cell again passing through a large to small cell transition (panels a and b) before apparently disappearing (or becoming 'silent') by day 30.

Characterization of suppressor cells generated in bone marrow reconstituted mice

The data above indicated that a highly significant early event in the regeneration of T cell function in irradiated mice, which occurred soon after the burst of T cell activity from a post-thymic precursor pool (day 15), was the generation of a transient cell population with suppressor cell properties. In view of the time sequence of these events, we thus wondered if the generation of these suppressor cells was dependent upon the existence of a thymus in the reconstituted mice. Accordingly, (C3H/He × AKR/J)F_1 mice were thymectomized (equal numbers were sham-thymectomized) and 2 weeks later inoculated with 10×10^6 cells per mouse from a pool of unfractionated bone marrow cells. At 20 and 25 days post-irradiation spleens of 5 mice from each group were pooled and 3×10^8 cells sedimented for 3 h at 4°. The relevant suppressor cell pools were prepared (see Fig. 6) and tested at graded doses for their ability to inhibit the development of CTL or SRBC-PFC from 5×10^5 or $2{\cdot}5 \times 10^6$ normal AKR/J spleen cells. Simultaneously, aliquots of each cell suspension prepared from sham thymectomized mice were treated with C3H anti-θ_{AKR} antiserum and complement, or by active adherence to plastic petri dishes (as described elsewhere) (Gorczynski, 1976b). The resultant T depleted and non-adherent populations, as well as the untreated sample were then again tested for their ability to inhibit CTL or PFC responses. These data are shown in Tables 2 and 3 respectively (one of three experiments).

It was apparent from Table 2 that no generation of suppressor cells took place in the thymectomized bone marrow reconstituted mice, despite the fact that these mice developed limited T cell responses from a post-thymic pool (Table 1). This indicated that the development of suppression observed above was dependent upon an intact thymus in the host (i.e., dependent in some fashion on a prethymic bone marrow precursor pool) and also that post-thymic precursor cells lacked the ability to generate suppressor cells (at least to a detectable frequency after *in vivo* inoculation assay as above). Moreover, as indicated in Table 3, the suppressor cells were themselves anti-θ sensitive, and non-adherent to plastic dishes. Thus the data of Fig. 6 could be interpreted in terms of the early regeneration of a non-adherent, suppressor, T cell pool which, like the functional T cells assayed in Figs 2–5, underwent a large cell to small cell transition after regeneration.

Table 3. Effect of anti-θ serum or active adherence to plastic on suppressor cells prepared from the sham-thymectomized bone marrow reconstituted mice of Table 2

Source of suppressor cells	Treatment of* suppressor cells	Cytotoxic† response per culture	SRBC-PFC‡ per culture
None	—	41 ± 3·6	168 ± 27
20 day reconstituted sham-thymectomized	None	4·8 ± 0·1	38 ± 7
	C3H anti-θ AKR	26 ± 2·9	106 ± 27
	adherence	2·8 ± 0·3	17 ± 5
25 day reconstituted sham-thymectomized	None	8·3 ± 1·1	42 ± 8
	C3H anti-θ AKR	29 ± 4·2	131 ± 15
	adherence	3·6 ± 0·7	31 ± 6

* See first footnote of Table 2. Only 4×10^5 of each treated pool of suppressor cells was tested here.

† and ‡ as for Table 2.

Table 4. Reconstitution of T cell functions from anti-Brθ treated bone marrow or spleen cells

Source of* tested cells	Day 15† CTL responses	Day 20‡ suppressor cells CTL response	Day 20‡ suppressor cells SRBC-PFC	Day 30 assays§ CTL responses	Day 30 assays§ SRBC-PFC helper T cells
Normal spleen	47 ± 4·8	38 ± 4·1	147 ± 22	40 ± 3·6	196 ± 38
Anti-Brθ spleen recipients	5·6 ± 0·7	2·9 ± 0·3	11 ± 4	13 ± 1·7	33 ± 5
Anti-Brθ bone marrow recipients	6·1 ± 0·8	8·4 ± 1·1	32 ± 6	36 ± 3·2	89 ± 17

* Spleen cells from these sources were tested in the various assays with or without prior sedimentation as described in the text.

† Per cent cytotoxicity from $7{\cdot}5 \times 10^5$ spleen cells assayed at day 15.

‡ Suppressor cells were obtained by sedimentation. For the CTL assay $5{\cdot}0 \times 10^5$ suppressor cells were mixed with $5{\cdot}0 \times 10^5$ normal spleen cells. For the PFC assay $5{\cdot}0 \times 10^5$ suppressor cells were incubated with $2{\cdot}5 \times 10^6$ normal spleen cells. The top line indicates the response from control cells with no suppressor cells added.

§ CTL response from $7{\cdot}5 \times 10^5$ spleen cells or SRBC-PFC response using 1×10^6 putative spleen helper T cells with $2{\cdot}0 \times 10^6$ anti-θ treated normal AKR/J spleen cells. The response of the latter alone was 8 ± 3.

Comparison of Anti-Brθ treated spleen and bone marrow as sources of T cell activities in lethally irradiated reconstituted mice

As a final analysis of the regeneration of different T cell functions from inactive cells *in vivo*, we compared the ability of mature T cell depleted (by anti-Brθ antiserum treatment) spleen and bone marrow cells to reconstitute irradiated (C3H/He × AKR/J)F_1 mice. Spleen and bone marrow cells were prepared from 10 normal AKR/J mice and the cells were treated with anti-Brθ and rabbit complement. 10×10^6 bone marrow or 7×10^7 spleen cells were then inoculated into 10 mice per group. At days 15, 20 and 30, spleen cells were pooled from 3 mice per group, as well as 3 fresh normal AKR/J mice, and the following assays performed:

(i) Day 15 cells: The cells were tested for their ability to generate CTL in culture with irradiated (BALB/c × AKR/J)F_1 spleen cells.

(ii) Day 20 cells: 2×10^8 of the cells were sedimented for 3 h at 4°. Cells in the region 6–9 mm/h were pooled and tested for their ability to suppress CTL or PFC from normal AKR/J spleen cells.

(iii) Day 30 cells: The cells were tested for their ability to generate CTL in culture and to help the SRBC-PFC response of anti-θ treated normal AKR/J spleen cells.

These data are shown in Table 4 (one of four experiments). There were three essential features of interest. As judged by the cytotoxic response at day 15, which we have demonstrated allows a quantitative assessment of post-thymic precursor cells injected at day 0 (see Fig. 4), the post-thymic pool of spleen cells was similar in size to that of bone marrow. Thus, when these precursor pools from spleen/bone marrow were injected in numbers representative of their recovery from normal mice ($1{\cdot}5 \times 10^8$ and 2×10^7 respectively), there was a similar recovery of progenitors of CTL in the spleen of the irradiated recipients at day 15 post-transplant. However, by day 20, it was apparent that the spleen thymus-dependent precursor pool generated more suppressor cells than the bone marrow precursor pool (auxiliary experiments established that the suppressor cells from spleen cells were again theta-positive cells). Finally, while by day 30 there was again no evidence for active suppressor cells in spleen cell reconstituted mice, just as in bone marrow reconstituted mice (Figs 5 and 6), yet the spleen reconstituted mice developed fewer progenitors of CTL and less T helper function (for antibody responses) than did bone marrow reconstituted animals. In view of the data of Fig. 4 showing the poor dose response curves for day 30 reconstitution, this suggested that the spleen was considerably depleted (relative to bone marrow) of prethymic precursor T stem cells.

DISCUSSION

A number of studies in recent years have generated evidence for the existence of distinct subpopulations of T lymphocytes with unique biological properties. Using those biological cell surface markers known to be possessed by T lymphocytes (e.g., theta antigen and Ly 1, 2, 3), it has been established that T helper cells, progenitors of cytotoxic cells and mitogen responsive cells can, to some degree, be distinguished from one another (Cantor *et al.*, 1975; Cantor & Boyse, 1975; Frelinger *et al.*, 1976). Similar separation is probably not possible by purely physical means with cell suspensions obtained from normal, healthy, adult mice (Shortman, 1974; Miller *et al.*, 1975).

Evidence has also been presented to indicate that the athymic (nu/nu) mouse (Ikehara, Hamashima & Masuda, 1975) and adult mouse bone marrow (Cohen & Patterson, 1975) can be induced to undergo maturation to cells with mature T-cell characteristics using various manipulations *in vivo* and *in vitro*. Since, where assayed, the biological functions assumed by the previously inactive T-precursor populations are of a complex nature (thought perhaps to involve more than one T-cell type), these data suggest that an indiscriminate differentiation of a number of T-cell subpopulations can be attained *in vitro*. This potential to induce and study the process of T cell differentiation wholly in tissue culture has profound implications not just for basic research but for clinical medicine also (e.g. tumor therapy, management of immunodeficiency diseases, etc.). Thus, it was deemed essential to understand more about the detailed differentiation of functionally active T cells from their inactive precursor(s), both *in vivo* and *in vitro*.

Approaching this problem from the standpoint of characterizing the physical nature of the reconstituting and reconstituted cell pools, we found evidence both for two independent precursor pools (prethymic and post-thymic—see Figs 3 and 4, and Table 2), and that the T cell functions we observed in regenerating mice could be ascribed to populations of cells with different sizes (Fig. 5). The ability to discriminate between T cells of different function by virtue of their physical size in bone marrow regenerating mice was most interesting. While all T cell functions seemed to appear first in large cells, which gradually reverted to small lymphocytes typical of those present in a normal spleen cell population, it seemed that at various times after regeneration some activities had already undergone this change while others persisted only in large cells (e.g., T helpers for SRBC-PFC at day 30, CTL progenitors at day 25, con A and MLC responding cells at day 20). One explanation may be that all T cells are programmed to express all functions at a discrete stage of maturation (delineated by distinct size changes). It seems to us more likely, however, that these differences represent the sequential regeneration, firstly in large cells, of those unique subpopulations known to exist in small resting T lymphocytes. Moreover, while the evidence on this point is rather less firm, there seems to be very little evidence for the belief that mature T-cell populations arise from different precursor pools. The sole contradiction to this is that the post-thymic precursor pool is apparently preferentially committed to very early development of PHA responding cells, and does not develop suppressor T-cells or helper cells (for antibody formation).

A note of caution must unfortunately be introduced regarding the kinetics of appearance at least of progenitors of CTL. Between days 18 and 28, there is undoubtedly a regeneration of a suppressor T cell pool capable of inhibiting the reactivity of normal spleen cells in either CTL or PFC assays. Given this fact, it is impossible to assign true kinetic activity curves, certainly to CTL progenitors; at these times there is little evidence to suggest that T-helpers for antibody responses are present and masked by suppressors However, since all other T cell functions investigated pass through the large cell to small cell transition described, we feel fairly confident in suggesting that the activity measured for progenitors of CTL at day 20 is an underestimate of the potential activity in the spleen at this time.

An interesting feature brought out best in Fig. 6 is that studies of populations in terms of their physical characteristics rather than surface properties often can give information not achieved by selective antiserum treatment. To date, it has not been possible to distinguish between T helpers for antibody formation and those responsible for help in the generation of cytotoxic cells. As indicated here (and in unpublished studies), it seems that the latter T helpers are indistinguishable in terms of size (at all times of regeneration) from those cells active in MLC proliferation assays and Con A responses, while helper cells for antibody function

are apparently a later regenerating T-cell population.

A final study we performed was to compare the organ distribution of the three potentially different types of T-cell reactivity we measured, namely the pre- and post-thymic T cell pools and the pool of precursors of suppressor T cells. Bone marrow and spleen cell suspensions as prepared from normal animals were found to contain relatively equal amounts of the post-thymic pool, while bone marrow was a better source of prethymic T precursors (and a poorer source of T suppressors) than the spleen. Since the precursor pool of T suppressors is apparently a prethymic one (see Table 2), this is perhaps the best evidence for subpopulations of precursor cells being responsible for the appearance of subpopulations of mature T cells.

The evidence for a post-thymic precursor pool, which matured within 15 days into cells with biologically detectable functional T cell activity in thymectomized mice, suggested that a reinterpretation of several phenomena may be in order. It is now open to possibility that the manipulations performed *in vitro* on adult bone marrow, and both *in vivo* and *in vitro* on nu/nu mouse cells may be directly affecting this post-thymic cell pool rather than triggering T cell differentiation *per se*. This question has been investigated in the following manuscript. A post-thymic pool in nu/nu could perhaps have appeared during that time in embryogenesis when the thymus was developing normally. Alternatively we ought perhaps to think instead in terms of a unique pool of progenitors of T cell subpopulations which can be totally independent of a thymic influence for development of those T cell subpopulations. Furthermore, the kinetics of appearance of reactivity from these two T-cell pools clearly parallels the kinetics of acute and chronic graft-versus-host disease seen on transplantation of rodent bone marrow across an H-2 barrier (Van Bekkum, & de Vries, 1976). It is thus tempting to speculate that those failures (due to acute GVH) of bone marrow transplantation in man, despite manipulation to ensure that no mature T cell function was transferred with the bone marrow, were caused by a failure to recognize and remove the post-thymic T-cell pool. If this is done so that the complications are more likely to be due to chronic GVH (caused by differentiation of the prethymic precursor pool), more successful transplantation may prove possible since it is known that chronic GVH is less severe and more amenable to conventional therapy (Balner, de Vries & Van Bekkum, 1964).

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