SEPARABLE POPULATIONS OF ACTIVATED THYMUS-DERIVED LYMPHOCYTES IDENTIFIED IN TWO ASSAYS FOR CELL-MEDIATED IMMUNITY TO MURINE TUMOR ALLOGRAFTS

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For many years specific immune responses have been generally classified as to whether they are mediated by humoral antibodies or by sensitized lymphoid cells. More recently has come the realization that on the cellular level these two types of responses are mediated by two major classes of lymphocytes, the bursal-equivalent or B cells being the precursors of antibody-forming cells and T cells being responsible for cell-mediated reactions (1–5). Immune responses within each of these major cell populations are in turn generally characterized by extensive heterogeneity (in terms of the products or activities eventually measured); a clearer understanding of the cellular basis for this heterogeneity is one of the major goals of current research.

Examination of the cellular heterogeneity within the B-cell pool following antigenic stimulation has been greatly facilitated by the availability of satisfactory techniques for the enumeration and study of isolated antibody-forming cells (6–9). Such studies have in general indicated that the specificity and immunoglobulin class of the product of an individual antibody-producing cell is highly restricted (10–14).

The absence of analogous single cell techniques for assaying the many activities generated within the T-cell pool following antigenic stimulation continues to restrict our ability to understand the cellular basis for this functional diversity. Until such methodology is available one can only approach the problem indirectly by examination and quantitation of the activities of different populations of T cells. If the quantitative analysis of the activities measured in these populations indicates a dissociation of activities, then it is reasonable to postulate that different cells mediate the activities in question. Such an hypothesis is then supported (indirectly) by any additional examples of situations in which these activities dissociate from each other.

In the present study we have investigated some of the physical and biological

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properties of the cells involved in two different assays of cell-mediated immunity generated by mice in response to the antigenic stimulation of a tumor allograft. The effector activities assayed were the cell-mediated lysis of radiolabeled target cells and the antigen-stimulated inhibition of macrophage migration. The results to be presented suggest that separable populations of T cells are involved in mediating these two reactions. The subpopulations have (*a*) distinctive tissue distributions in appropriately immunized animals, (*b*) differential migratory properties upon adoptive transfer into irradiated syngeneic hosts, and (*c*) distinctive activity profiles after 1 *g* velocity sedimentation. Additional experiments utilizing velocity sedimentation of normal spleen cells before their in vitro sensitization to alloantigens further suggest that the progenitors of these T-effector cells may be different.

Materials and Methods

Mice.—8–12-wk old male mice of the strains BALB/c ($H\text{-}2^d$), DBA/2 ($H\text{-}2^d$), and C57BL/Blue ($H\text{-}2^b$) were obtained from the Imperial Cancer Research Fund breeding unit at Mill Hill, London.

Irradiation.—Mice were irradiated by gamma rays from a Co^{60} source with a total of 850 rads at a dose rate of 24 rads/min. When irradiated cells were needed, cell suspensions were kept chilled on ice and exposed to 1,500 rads, at a dose rate of 200 rads/min.

Cell Preparation.—Spleen, mesenteric lymph node (MLN),[1] or peripheral lymph node (PLN, cervical, submandibular, axillary and inguinal) cells were prepared by teasing the respective tissues apart in ice-cold phosphate-buffered saline, pH 7.2 (PBS). Large fragments were allowed to settle out and the supernatant suspensions were centrifuged at 200 *g* for 10 min at 4°C. Peripheral blood was collected into heparinized PBS (10 U/ml) and the suspension centrifuged at 200 *g* for 10 min at 4°C. Red cells in all of the above suspensions were lysed with Tris-buffered ammonium chloride, according to the method described by Boyle (15). Following this treatment the suspensions were washed three times in cold PBS. Peritoneal macrophages were obtained from C57BL mice injected intraperitoneally (i.p.) 2–4 days earlier with 0.25 ml complete Freund's adjuvant (16). Before use, all cell suspensions were resuspended to the desired concentration in Dulbecco's modified Eagle's medium (with added glutamine, penicillin, and streptomycin) supplemented with 10% heat-inactivated fetal calf serum (DF_{10}). All cell concentrations refer to viable nucleated cells, as determined by trypan blue dye exclusion.

Preparation, Specificity, and Use of Rabbit Antimouse Brain-Associated θ Serum (Anti-BAθ).—Anti-BAθ serum was prepared as described by Golub (17). The antiserum was heat-inactivated at 56°C for 30 min and was then absorbed twice for 30 min at room temperature with an equal volume of packed BALB/c liver suspension; finally it was filtered through a Millipore filter (0.45 μ) and stored at −20°C.

Cells to be treated with the anti-BAθ serum were incubated for 90 min at 4°C at a concentration of 10^7 cells/ml in a 1/15 dilution of the antiserum diluted in DF_{10}. Control suspensions were incubated in DF_{10} alone. The cells were then washed in DF_{10}, centrifuged at 200 *g*

[1] *Abbreviations used in this paper:* anti-BAθ, rabbit antimouse brain-associated θ; BSA, bovine serum albumin; con A, concanavalin A; DF_{10}, Dulbecco's modified Eagle's medium + 10% fetal calf serum; DNP-POL, dinitrophenylated polymerized flagellin; EBSS, Earle's balanced salt solution; LPS, *E. Coli* lipopolysaccharide; MLN, mesenteric lymph nodes; PBL, peripheral blood leucocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PLN, peripheral lymph nodes; SMAF, specific macrophage-arming factor.

for 5 min at 4°C and resuspended to the same volume in either DF_{10} or fresh frozen guinea pig serum (1/10 in DF_{10}) which had been previously absorbed twice with 10% packed mouse thymocytes. The suspensions were incubated at 37°C for 45 min in an atmosphere of humidified 10% CO_2 in air, washed twice, and then resuspended to the desired concentration in DF_{10}.

Detailed specificity tests of this antiserum have been previously described (18). Briefly, three types of tests have been employed. The serum was examined for its cytotoxicity (determined by trypan blue dye exclusion) toward normal mouse thymus and PLN cells. In the absence of complement the serum was not toxic for these cells, and in the presence of complement killed 100% of thymocytes and 70% of PLN cells, this data being in good agreement with previously published percentages of T cells in such suspensions (19). A second test of the functional specificity of this antiserum toward T cells examined its ability to suppress the enhanced protein synthesis (measured by incorporation of [^{3}H]leucine) seen in suspensions of spleen cells cultured for 18–24 h in leucine-free medium with either PHA or *Escherichia coli* lipopolysaccharide (LPS), T and B cell mitogens, respectively (20). Pretreatment of spleen cells with the anti-BAθ serum and complement abolished the PHA response, but left the LPS response unaffected. The third test employed was the effect of antiserum and complement pretreatment of cells on their ability to subsequently make antibody-forming cells in vitro to sheep erythrocytes (SRBC) and dinitrophenylated polymerized flagellin (DNP-POL). Such pretreatment abolished the SRBC response but had no effect on the response to DNP-POL, the latter presumably being a T-cell independent response (21). Furthermore, the depleted SRBC response of the treated spleen cells was fully reconstituted with small numbers of educated thymocytes (obtained from spleens of mice irradiated with 850 rads 7 days earlier and then immediately given 10^8 SRBC and 10^8 syngeneic thymocytes), but was not reconstituted with mature B cells (obtained from those lymphocytes of adult mouse bone marrow which sediment by velocity sedimentation at 2.7–3.3 mm/h (18, 22, 23).

Tumor Cell Lines.—P815X2, an ascites tumor of mast cells derived from DBA/2 mice (24), was obtained from Dr. J. C. Cerottini and was maintained in vivo by serial weekly i.p. passage in DBA/2 mice. This cell line was also maintained in vitro by serial passage in DF_{10} at 37°C in a humidified atmosphere of 10% CO_2. Cell concentration was kept at $<2 \times 10^6$ cells/ml (5 ml of cell suspension in 60 x 15-mm tissue culture dishes); under these conditions a doubling time of 10–12 h was routinely seen and the harvested cells provided excellent reproducible targets for the cytotoxicity assay described below. Every 12–18 wk aseptically aspirated ascitic fluid from tumor-bearing mice was used to derive a fresh tissue culture cell line. In some experiments the C57BL ascites tumor EL-4 (25) was used as a target cell. This tumor was the gift of Dr. J. Martin and was maintained by weekly serial passage in C57BL mice.

In Vivo Immunization.—P815X2 cells were obtained from the peritoneal cavity of tumor-bearing DBA-2 mice by collecting an aspirate into PBS containing 10 U/ml heparin. The cells were washed three times and then counted. Ten million viable tumor cells (in 0.5 ml) were then injected i.p. into each of a number of C57BL mice, care being taken to avoid subcutaneous inoculation.

In Vitro Immunization.—Seven million normal unfractionated or velocity-sedimented (see below) C57BL spleen cells were cultured in the presence of 3×10^6 irradiated (1,500 rads) BALB/c spleen cells in flat-bottom plastic tubes (12 mm diameter) in 4 ml of DF_{10} containing 5×10^{-5} M 2-mercaptoethanol. Multiple identical culture vessels were set up for each fraction to be tested, the exact number depending on the numbers of cells available from that fraction. After 5 days incubation in a stationary position at 37°C in a humidified atmosphere of 10% CO_2 in air, the multiple cultures were harvested (all the cultures from a given fraction were pooled), washed once, resuspended to appropriate volumes with DF_{10}, and the aliquots tested in both the cytotoxicity assay and the macrophage migration inhibition assay (see Results).

Velocity Sedimentation Cell Separation.—This technique, which separates cells primarily on the basis of cell size, has been described in detail elsewhere (22). Sterile glass sedimentation

chambers, 11.0 cm in diameter (Aimer Glass Co., London, England) were used in all experiments. Briefly, 3×10^8 nucleated spleen cells (or 2×10^8 PLN cells) were suspended in 30 ml of 0.3% bovine serum albumin (BSA) in Earle's balanced salt solution (EBSS) and separated at unit gravity for 3 or 4 h at 4°C through a buffered step gradient of BSA in EBSS ranging in concentration from 0.6% to 2.0%. 30-ml fractions were collected, the cells in each fraction counted, and the suspensions centrifuged at 200 *g* for 10 min at 4°C. Appropriate fractions were pooled (see Results), washed again, recounted, and resuspended to appropriate volumes in DF_{10}. Yields were generally 75–90% of the cells initially loaded. The osmolarity of the EBSS and Dulbecco's modified Eagle's medium used throughout these experiments was approximately 270 mOsmol/liter and 370 mOsmol/liter, respectively.

Cytotoxicity Assay.—Five–ten million P815X2 cells were radiolabeled in 1.0 ml DF_{10} containing 50 μc ^{51}Cr (as sodium chromate, Radiochemical Centre, Amersham, England) for 40 min at 37°C in 10% CO_2 in air. After incubation the suspension was washed four times in 25 ml of room temperature Dulbecco's medium containing 20% fetal calf serum by centrifugation at room temperature for 5 min at 200 *g*. These target cells were then resuspended in DF_{10} to 5×10^5 cells/ml. Various numbers of viable immune cells in 1.0 ml DF_{10} were then mixed with 5×10^4 ^{51}Cr-labeled P815X2 cells (0.1 ml) in 16-mm tissue culture wells. Any group was always assayed in triplicate. After incubation on a rocking platform (6 cycles/min) at 37°C in a humidified atmosphere of 10% CO_2, the contents of the wells were harvested, centrifuged at 1,000 *g* for 10 min, and the supernatant fluid counted in a well-type gamma counter. Controls included target cells incubated with either medium alone (background) or with 5% detergent (maximum releasable ^{51}Cr). Detergent release was routinely 75–80% of the total ^{51}Cr in the suspension. Several early experiments also utilized various numbers of normal cells from spleen, MLN, and PLN incubated with target cells. As was noted by Brunner et al. (26), the release in the presence of normal lymphocytes was not significantly different from the release in the presence of medium alone, and thus for convenience the latter control was routinely used for determination of background ^{51}Cr release. This background varied from 8–17% (mean 12.8%) during the several months when these experiments were being performed. Specific ^{51}Cr release was calculated according to the formula: specific ^{51}Cr release (%) = 100 × (experimental-background/maximum releasable ^{51}Cr-background). Data points in text are means ± 1 standard error of the mean (SEM) of triplicate determinations.

Macrophage Migration Inhibition Assay.—A modification of earlier techniques (27), which has been found to be suitable for use in mice, has been described previously in some detail (16). Normal mouse macrophages were obtained as peritoneal exudates from C57BL mice injected 2–4 days earlier with 0.25 ml complete Freund's adjuvant, washed, and resuspended to 10^8 cells/ml (in DF_{10}). 0.04 ml of this suspension was then mixed with 0.04 ml of immune or normal C57BL cell suspension (see Results for specific cell number) and 0.02 ml of either irradiated (1,500 rads) BALB/c spleen cells as specific antigen or irradiated C57BL spleen cells as controls (each at 10^8/ml). Capillary tubes (25 μl) were filled with the suspension, flame sealed at one end, and spun at 200 *g* for 5 min at 4°C. The tubes were cut at the cell-fluid interface and the stubs fixed with a drop of silicone grease in 35-mm tissue culture dishes; 2 ml of DF_{10} were then added to each dish and the dishes incubated at 37°C in 10% CO_2 for 18–24 h. The area of migration was quantitated using a dissecting microscope fitted with an eyepiece graticule. The percent migration inhibition was calculated as:

$$100 \times \left(1 - \frac{\text{mean migration in presence of specific antigen}}{\text{mean migration in absence of specific antigen}}\right).$$

Four capillaries were used for each group; data is expressed as mean % migration inhibition ± 95% confidence limits.

RESULTS

Tissue Distribution of Cytotoxic Lymphocytes at Various Times After Immunization.—C57BL mice were immunized by i.p. inoculation of 10^7 P815X2

cells. At various times thereafter groups of mice were sacrificed and cell suspensions of various tissues prepared. Any single experiment involved pools of cells harvested from at least four mice. Separate suspensions were prepared from MLN and PLN (i.e. axillary, cervical, submandibular and inguinal). These suspensions, as well as suspensions of spleen cells and peripheral blood leucocytes (PBL) were then tested for their capacity to lyse ^{51}Cr-labeled P815X2 cells. The results of these experiments are summarized in Fig. 1.

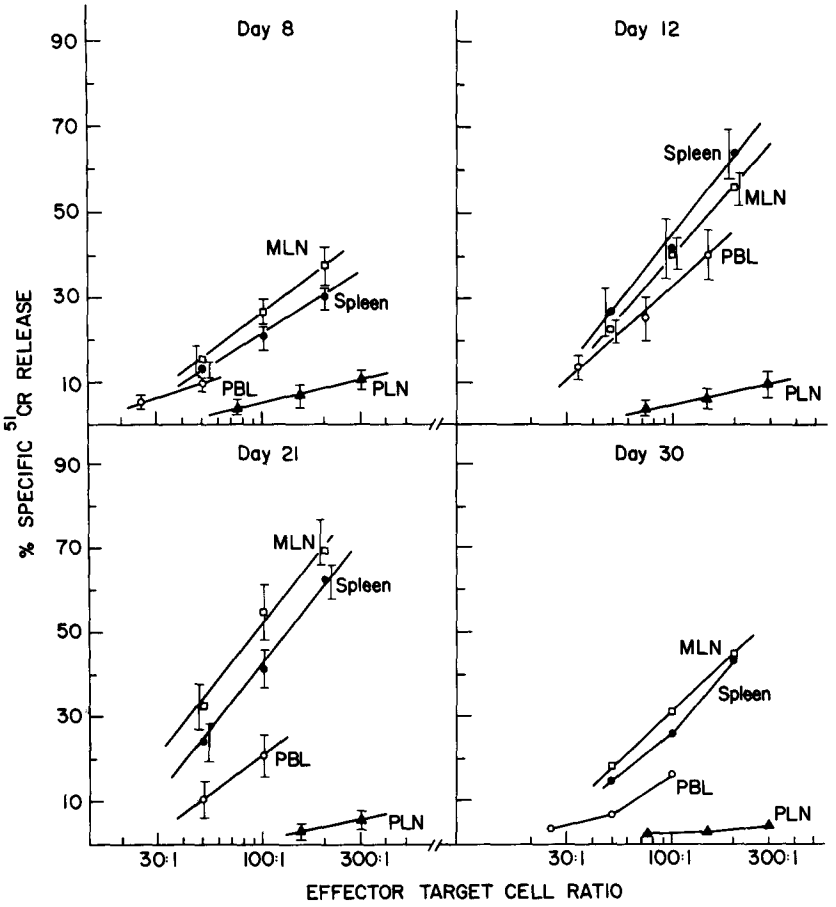

FIG. 1. Cytotoxicity for ^{51}Cr-labeled P815X2 targets of various lymphoid tissues harvested from C57BL mice at different times after i.p. inoculation of 10^7 allogeneic tumor cells (P815X2). Tissues examined were PLN (▲), PBL (○), MLN (□), and spleen (●). All wells contained 5×10^4 ^{51}Cr-labeled P815X2 cells and the indicated number of viable immune cells; incubation was carried out for 4 h at 37°C on a rocking platform. In any single experiment, each dilution of immune cells was plated in triplicate and the results calculated as mean % specific ^{51}Cr release (see Materials and Methods). The data shown for day 30 are results from a single experiment; that for days 8, 12, and 21 are means ± 1 SEM calculated from data pooled from at least three separate experiments for each of the indicated points.

Several observations warrant comment. The activity of any population was a linear function of the logarithm of the number of attacking cells present in the incubation mixture, an observation noted by several previous investigators (28–30). Spleen, MLN, and PBL cells showed an obvious increase in activity between day 8 and day 12, indicated both by a shift in the dilution curves to the left as well as by an increase in the slope of the dilution curves (28). The levels of cytotoxic activity in these three tissues remained at relatively high levels through day 21, although PBL seemed to have decreased somewhat by this time. By day 30 there was a decrease in the cytotoxic activity of these three tissues. These results are very similar to those previously reported by Brunner et al (26).

In contrast to the substantial levels of cytotoxicity observed in spleen, MLN, and PBL, PLN cells displayed very little cytotoxic activity at any of the times examined. Very low levels were seen on day 8 (11.5% lysis at a 300:1 ratio) and this activity showed no tendency to increase with time after immunization. Although accurate quantitation of the differences between PLN and the other tissues is difficult because of the obvious nonparallelism of the dilution curves, nevertheless one can estimate that at any of the times tested PLN cells were approximately only $\frac{1}{10}$th as active as spleen, MLN, or even PBL cells.

These results suggested a marked deficiency in cytotoxic lymphocytes in the peripheral lymph nodes of mice immunized i.p. with a tumor allograft, when such cells were compared to the other tissues tested. However an alternate explanation for these observations was that in addition to containing substantial numbers of cytotoxic lymphocytes, PLN suspensions also contained cells and/or substances which directly suppressed the expression of this cytotoxicity. If such suppressive activity were present in PLN suspensions, it might be possible for these suspensions to directly inhibit the cytotoxic activity of populations known to be active. Direct evidence for such suppressive activity in PLN suspensions was sought by mixing 12 day immune PLN cells with either 12 day immune spleen, MLN or PBL cells for 1 h at 37°C before testing for cytotoxic activity. Control suspensions of unmixed populations were also incubated before testing. Table I demonstrates that under the conditions tested, no such suppressive activity was found. The cytotoxic activity of either 2.5×10^6 PBL or 5×10^6 spleen or MLN cells was identical whether preincubated and tested in medium alone or in medium containing 5×10^6 immune PLN cells.

It seemed likely that the route of administration of the immunizing tumor allograft was a major factor in determining the relative levels of cytotoxic activity observed. In order to test this more directly, C57BL mice were inoculated in several subcutaneous sites (instead of i.p.) with a total of 10^7 P815X2 cells. 14 days later suspensions of PBL, spleen, MLN, and PLN cells from these mice were tested in the cytotoxicity assay. The results of this experiment

TABLE I

Lack of Demonstrable Suppressive Activity in 12-Day Immune PLN Cells on the Cytotoxic Activity of 12-Day Immune PBL, MLN, or Spleen Cells

Cell source	% Specific ^{51}Cr Release*	
	Alone	With added PLN‡
PLN (100:1)§	5.5 ± 0.8	—
PBL (50:1)	26.5 ± 0.8	27 ± 0.3
MLN (100:1)	37 ± 1.2	38 ± 1.5
Spleen (100:1)	53 ± 1.3	55 ± 0.6

* Calculated as described in Materials and Methods. Numbers are means of triplicate determinations ± SEM.

‡ Immune PLN cells were preincubated with the population to be tested for 1 h (37°C) before incubation with target cells. Each well contained 5×10^6 (100:1) PLN cells in addition to the indicated number (see §) of either PBL, MLN, or spleen cells.

§ Effector:target cell ratio; all wells contained 5×10^4 ^{51}Cr-labeled P815 cells.

showed that after this route of immunization, PBL, spleen, and PLN cells showed substantial levels of cytotoxicity (approximately 40%, 70%, and 30% specific lysis, respectively, at ratios of 200:1) while now MLN cells were virtually inactive (only 8% lysis at a 200:1 ratio).

Taken together, the above results suggested that both PLN and MLN could generate cytotoxic activity but that proximity of antigen was crucial in this generation. Distal, presumably nondraining lymph nodes would neither generate nor contain substantial cytotoxic activity; on the other hand PBL (and spleen) contained substantial cytotoxic activity irrespective of the route of antigen administration. This discrepancy between proximal lymph nodes and PBL on the one hand and nondraining lymph nodes on the other suggested that cytotoxic lymphocytes were not equally distributed throughout the recirculating pool (31); their presence in PBL did not insure access into lymph node tissue.

Other investigators using similar models for generating and assaying cytotoxic activity to tumor allografts have examined in some detail the specificity of target cell lysis (30, 32–34). They have concluded that lysis of target cells other than the one used for immunization can for the greatest part be explained by sharing of serologically defined *H-2* antigenic specificities. Detailed specificity tests were not performed in this study. Nevertheless, two experiments were performed in which the specificity of the cytotoxicity assay was examined. 14-day C57BL anti-P815X2 immune spleen cells were tested for their capacity to lyse either ^{51}Cr-labeled P815X2 cells or ^{51}Cr-labeled EL-4 cells, a C57BL ascites tumor. Additional wells were set up with the immune C57BL spleen cells, ^{51}Cr-labeled EL-4 cells, as well as unlabeled P815X2 cells. Five million immune spleen cells were able to release 42% of the ^{51}Cr when incubated with 5×10^4 labeled P815X2 cells, but caused only 1% specific lysis of 5×10^4

labeled EL-4 cells and only 1.5% specific lysis from 5×10^4 labeled EL-4 cells in the presence of 5×10^4 unlabeled P815X2 cells. This absence of "bystander" lysis had also been observed by other investigators (30, 32, 33).

Comparison of Immune Spleen and PLN Cells in Tests of Cytotoxicity and Macrophage Migration Inhibition.—Having demonstrated that PLN cells of i.p. immunized mice were markedly deficient in cytotoxic lymphocytes compared to other tissues, it was of interest to determine whether this deficiency would also be apparent if one used another assay for cell-mediated immunity, namely, the ability of sensitized lymphocytes in the presence of specific antigen to inhibit the migration of mouse peritoneal macrophages. This assay has previously been used as a measure of cell-mediated immunity of mice to both alloantigens (16) and Maloney sarcoma virus (35, 36). Accordingly, suspensions of spleen and PLN cells were prepared from C57BL mice 14 days after i.p. inoculation with 10^7 P815X2 cells. Aliquots of these cells were then tested in parallel for both cytotoxicity and capacity to inhibit macrophage migration. These results are shown in Fig. 2. As in previous experiments spleen cells were found to be vastly superior to equal numbers of PLN cells in their capacity to lyse ^{51}Cr-labeled P815X2 cells (panel *a*). On the other hand, as shown in panel *b*, immune PLN cells were at least as active on a cell for cell basis as immune spleen cells in the assay of macrophage migration inhibition. It can be seen that in the presence of specific antigen (irradiated BALB/c spleen cells) the % migration inhibition with either immune population was a linear function of the number of immune cells in the capillary tube. In the absence of specific antigen neither population showed significant migration inhibition when compared to the migration of macrophages mixed with normal C57BL spleen cells either with or without specific antigen. (These latter controls were included in this and all subsequent experiments and are always represented by 0% migration inhibition; i.e., all experimental groups were compared to this figure in calculating the data.) Although irradiated BALB/c spleen cells were routinely used as the specific antigen in this assay, similar quantitative results have been found whenever irradiated DBA/2 spleen cells were used.

These results showed that although immune PLN cells were markedly deficient in cytotoxic lymphocytes when compared to immune spleen cells, they were not at all inferior to spleen cells when tested in the migration inhibition assay. That is, while substantial amounts of both activities were present in immune spleen cell suspensions, immune PLN cells had significant amounts only of migration inhibition activity. This dissociation was the first suggestion that different cells might be mediating these two effector activities.

Adoptive Transfer of Immune Spleen Cells into Lethally Irradiated Syngeneic Recipients.—The experiments illustrated in Fig. 2 suggested to us that different cells might be mediating cytotoxicity and macrophage migration inhibition. If this hypothesis was correct, then one might anticipate that upon adoptive transfer (into syngeneic recipients) of an immune population known to contain

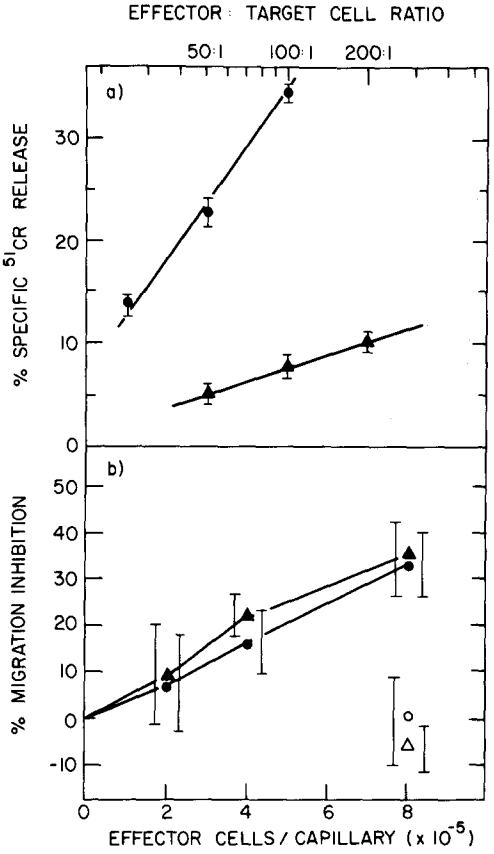

FIG. 2. Quantitative comparison of spleen (●) and PLN (▲) cells from C57BL mice 14 days after i.p. immunization with 10^7 P815X2 cells in two in vitro assays of celi-mediated immunity. Panel *a* illustrates the cytotoxicity of these immune cells for ^{51}Cr-labeled P815X2 cells. Test performed and results calculated as described in Materials and Methods. Panel *b* shows the data obtained in parallel in a test of macrophage migration inhibition. Closed symbols show the % migration inhibition of 10^6 C57BL macrophages seen with the indicated numbers of immune cells in the presence of specific antigen (4×10^5 irradiated BALB/c spleen cells), the open symbols that seen in the absence of antigen (4×10^5 irradiated C57BL spleen cells). Data points represent the arithmetic means, with 95% confidence limits, of quadruplicate determinations.

substantial amounts of both effector activities these effector activities might display different patterns of localization in recipient lymphoid tissues. The next series of experiments were performed to test more explicitly this hypothesis. Spleen cell suspensions were prepared from C57BL mice immunized i.p. 14 days earlier. These cells were then injected intravenously into normal C57BL recipients (10^8 cells/recipient) who had 2 h previously been exposed to 850 rads. 24 h later these mice were sacrificed and suspensions prepared from

either the pooled spleens or pooled peripheral lymph nodes. Viable nucleated cell counts revealed that the yield of spleen cells was approximately 12×10^6 cells/recipient and of PLN cells approximately 2.5×10^6 cells/recipient. These suspensions were then each tested in both the cytotoxicity assay and migration inhibition assay; the results are depicted in Fig. 3. The upper panel shows that on a cell for cell basis the spleen seeking (SpS) cells were approximately 10 times as active as the lymph node seeking (LNS) cells in lysing ^{51}Cr-labeled P815X2 cells. In contrast to these findings, when the cells were tested for their capacity to inhibit macrophage migration (lower panel), the LNS cells were found to be at least twice as active on a cell for cell basis as the SpS population. These results were confirmed on two additional occasions.

An aliquot of the original 14 day immune spleen cell suspension had been labeled in vitro with ^{51}CR. These cells were similarly injected into lethally irradiated syngeneic recipients; 24 h later the mice were sacrificed and various tissues removed and counted in a gamma counter to determine the % localization of the injected cells. The mean % localization ($\pm$ 1 SEM) was determined for the following tissues: spleen, 42 $\pm$ 3.6%; PLN, 5.4 $\pm$ 1.2%; MLN, 6.5 $\pm$ 1.1%; liver, 40 $\pm$ 0.9%; lung, 3.0 $\pm$ 0.5%; and small intestine, 2.6 $\pm$ 0.7%. This homing pattern of immune spleen cells is in good agreement with previously published studies (37, 38) of normal spleen cells, and argues that the distinctive migration patterns of effector activities described above was not due to abnormal preparation or handling of the cell suspensions.

The results of these experiments showed that while cytotoxic cells displayed a decided preference to migrate to the spleens rather than to the PLN of irradiated syngeneic recipients, the cells capable of effecting macrophage migration inhibition were much more able to localize in or migrate to PLN. Such findings support the hypothesis that the cells mediating these two reactions are not identical.

Effect of Anti-T-Cell Antiserum Pretreatment of Immune Cells on Cytotoxic and Macrophage Migration Inhibition Activities.—Several investigators have concluded that the cytotoxicity observed in a short term ^{51}Cr release assay with cells immunized to alloantigens either in vivo (33, 39) or in vitro (40) is due principally to T-cell-mediated lysis, although other cell types have been implicated (41–44). Similarly, the cell type which mediates antigen-specific macrophage migration inhibition has characteristics of a T cell in both the guinea pig (45–47) and the mouse (16, 35, 36). Nevertheless, because of our observations suggesting that different cells might be mediating these reactions, we felt it was important to determine the derivation of the effector cells involved in our particular assays. This was studied by investigating the effects of in vitro pretreatment of immune cells with a rabbit antimouse brain antiserum (21). The specificity tests performed with this antiserum have been described in detail elsewhere (18) and are summarized in Materials and Methods. By all criteria examined this antiserum in the presence of guinea pig com-

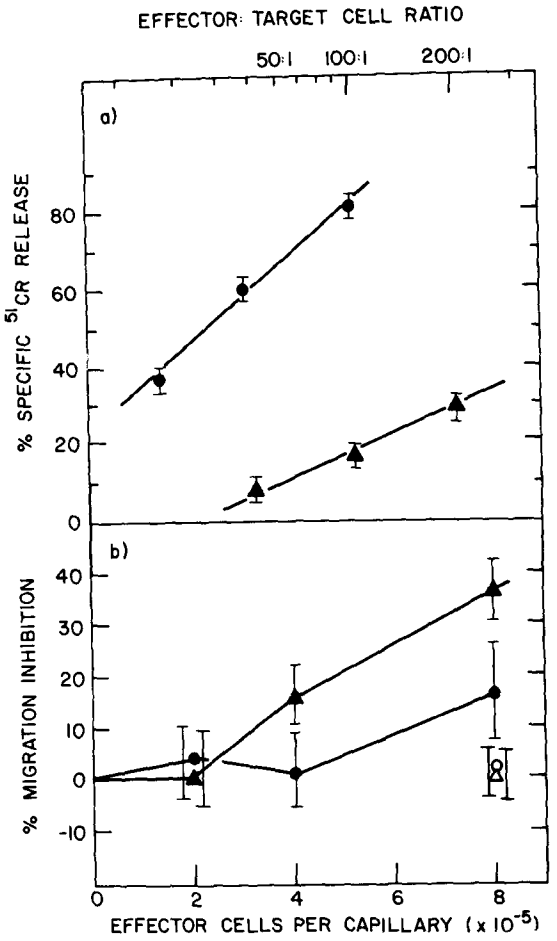

FIG. 3. Adoptive transfer of 14-day immune spleen cells into lethally irradiated syngeneic recipients: quantitative comparison of the cytotoxic and macrophage migration inhibition activities found in cells recovered from recipient spleens (●) and PLN (▲) 24 h later. 10^8 immune spleen cells from mice immunized 14 days before with 10^7 allogeneic tumor cells were transferred into each of several C57BL recipients exposed 2 h previously to 850 rads. 24 hours later the animals were sacrificed and suspensions prepared from spleens and PLN. Panel *a* illustrates the capacity of various numbers of such cells to lyse ^{51}Cr-labeled P815X2 cells (5×10^4 targets/well). Results expressed as means (± 1 SEM) of triplicate determinations. Panel *b* shows the results of the parallel test of various numbers of such cells to inhibit macrophage migration. Tubes contained 10^6 macrophages, the indicated numbers of immune cells, and either 4×10^5 irradiated BALB/c spleen cells (closed symbols) or 4×10^5 irradiated C57BL spleen cells (open symbols). Data points are means (±95% confidence limits) of quadruplicate determinations assayed after 18 h.

plement is cytotoxic only for T lymphocytes. Table II shows the results of pretreatment of 14 day immune spleen cells with this antiserum either in the presence or absence of guinea pig complement (GPC). Untreated cells exhibited substantial activity in both assays. Neither antiserum alone nor complement alone significantly affected these results. However pretreatment with both antiserum and complement essentially completely abolished both cytotoxic and migration inhibitory activities from the suspensions. Additional experiments (data not shown) also indicated that the cytotoxic activity of immune PBL was similarly eliminated by this pretreatment. We concluded that the

TABLE II

Effect of Anti-BAθ Serum on Effector Cell Activity of 14-Day Immune Spleen Cells in Assays of Cytotoxicity and Migration Inhibition

Pretreatment of cells*	Specific ^{51}Cr release‡	Migration inhibition§
	%	%
None	76 ± 2.0	35 ± 12
Anti-BAθ alone	82 ± 1.3	38 ± 8
GPC alone	78 ± 1.6	34 ± 8
Anti-BAθ + GPC	5 ± 2.0	6 ± 5

* Preparation of antiserum, specificity tests and treatment of cells with antiserum and complement are described in Materials and Methods.

‡ Mean % specific ^{51}Cr release ± 1 SEM (triplicate determinations). After each of indicated pretreatments, cells were resuspended so that each well contained 7.5×10^6 viable immune spleen cells (effector:target cell ratio, 150:1).

§ Mean % migration inhibition ± 95% confidence limits (quadruplicate determinations). After each of indicated pretreatments, cells were resuspended so that each capillary tube contained 8×10^5 viable immune spleen cells, 1×10^6 C57BL macrophages and either 4×10^5 irradiated BALB/c spleen cells or 4×10^5 irradiated C57BL spleen cells.

cytotoxic and macrophage migration inhibition assays in the present model were indeed both primarily assays of T-cell-mediated immune reactions.

Velocity Sedimentation Profiles of Immune Spleen and PLN Cells Active in Cytotoxicity and Migration Inhibition.—The next experiments were carried out to investigate whether the T cells mediating these two reactions might have distinctive physical properties in addition to the distinctive in vivo characteristics described above. To do this we utilized the technique of velocity sedimentation, which separates cells principally on the basis of size (22). 14-day immune spleen or PLN cells were sedimented at 1 *g* for 3 h at 4°C through a buffered step BSA gradient. Fractions were collected, washed and resuspended to equal volumes. The two upper panels in Fig. 4 (*a* and *b*) show the viable nucleated cell profiles for these velocity sedimented immune spleen and PLN cells, each exhibiting the expected peak of cells sedimenting at about 3 mm/h, and rapidly falling off, with only about 5% of the cells sedimenting at more

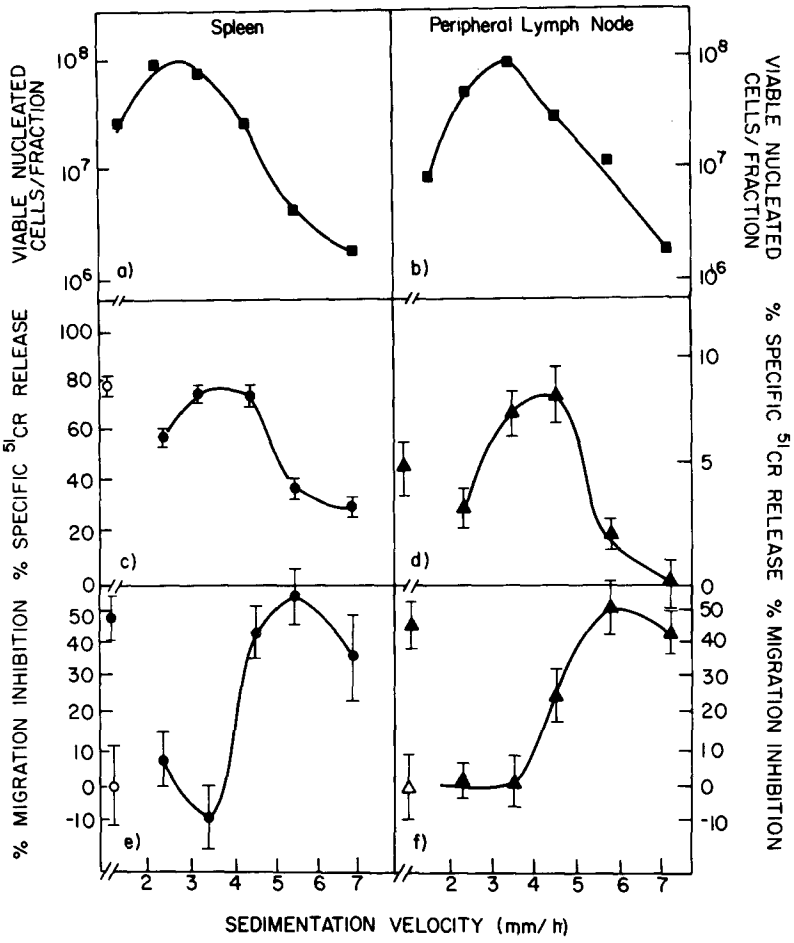

FIG. 4. Velocity sedimentation properties of immune spleen and PLN cells active in cytotoxicity and macrophage migration inhibition. Spleen and PLN cells obtained from C57BL mice 14 days after i.p. inoculation with 10^7 P815X2 cells. Both populations subjected to velocity sedimentation (see Materials and Methods for details) for 3 h at 4°C. Panels *a* and *b* show the sedimentation profiles of viable nucleated cells from spleen and PLN, respectively. The fractions were each resuspended to equal volumes, and constant percentages of each fraction then tested for their capacity to lyse ^{51}Cr-labeled P815X2 cells and to inhibit macrophage migration. Panels *c* and *d* show results of cytotoxicity assay using velocity sedimented spleen (●) and PLN (▲) cells. Data points are means (±1 SEM) of triplicate determinations, each well containing 5×10^4 target cells and 10% of the cells from a given fraction. Data points at far left of panels *c* and *d* show the mean % specific lysis of these targets by 5×10^6 unfractionated immune spleen cells and 1.25×10^7 unfractionated immune PLN cells, respectively. Panels *e* and *f* show the migration inhibition activity of velocity sedimented spleen (●) and PLN (▲) cells. Data points are means (±95% confidence limits) of quadruplicate determination, each capillary tube containing 10^6 macrophages, 1% of the cells contained in a given fraction, and 4×10^5 irradiated BALB/c spleen cells as antigen. Data points at far left of each panel indicate the inhibition seen with 8×10^5 unfractionated immune cells/tube in the presence of either 4×10^5 irradiated BALB/c spleen cells (closed symbols) or irradiated C57BL spleen cells (open symbols).

than 6 mm/h. Constant percentages of each fraction were then tested in parallel in either the cytotoxicity or migration inhibition assays.

The left half of Fig. 4 depicts the results obtained with fractionated immune spleen cells. Panel *c* shows that the majority of 14 day splenic cytotoxic activity was associated with cells which sedimented at less than 4 mm/h; on the other hand, as shown in panel *e*, virtually all of the antigen-stimulated migration inhibition activity localized with cells sedimenting at more than 4 mm/h. The right half of Fig. 4 shows that very similar results were obtained with velocity-sedimented immune PLN cells. What little cytotoxic activity was present in PLN cells was found to sediment with the cells having sedimentation velocities of 4.5 mm/h or less while the macrophage migration inhibition activity was all found in fractions containing cells which sedimented at > 4.5 mm/h. The use of such activity profiles to analyze the sedimentation velocities of cells with a given effector function is valid only if the linear dilution curves for the various fractions do not have grossly disparate slopes (i.e., plots of effector activity as a function of the number of immune cells). This was confirmed (data not shown) for cytotoxicity in all experiments. Thus although for convenience the data points shown in Fig. 4 were those obtained with a single dilution of effector cells, all fractions were tested at at least three separate dilutions; plotting this data as % specific lysis versus the log of the number of immune fractionated cells yielded a series of straight and relatively parallel lines.

These experiments showed that under the conditions employed one could achieve at least partial separation of immune T cells mediating either cytotoxicity or macrophage migration inhibition on the basis of cell size; when tested directly after velocity sedimentation cytotoxic activity localized predominantly with small lymphocyte-enriched fractions whereas migration inhibition activity localized predominantly in fractions enriched with larger lymphocytes.

Velocity Sedimentation Profile of Splenic Progenitors of Cells Active in Cytotoxicity and Migration Inhibition.—The previous experiments had each given results compatible with the hypothesis that the T effector cells for cytotoxicity and macrophage migration inhibition belonged to different subpopulations of activated T cells. If this were so, then these subpopulations could have been generated either from a common precursor population or alternatively from distinct subpopulations of precursor cells. An important additional question in this latter case is at what point in time and space does this commitment to a given function take place? In a preliminary attempt to approach this question we asked whether there was any difference between the velocity sedimentation profile of the splenic precursors of cytotoxic lymphocytes and the profile of the splenic precursors of migration inhibitory lymphocytes. Accordingly, normal C57BL spleen cells were subjected to velocity sedimentation for 4 h at 4°C. The time of sedimentation was increased in these experiments in order to allow more accurate collection of fractions of cells which had rather small differences

in sedimentation rates. The fractions collected are shown in the top panel of Fig. 5. Multiple cultures of each fraction were then set up as described in Materials and Methods with 7×10^6 fractionated spleen cells and 3×10^6 irradiated BALB/c spleen cells as antigen. The number of cultures initiated for any fraction was of course limited by the number of cells in that fraction. After 5 days the cultures were harvested, those from a given fraction pooled and washed once; all groups were then resuspended in similar volumes of DF_{10}. Constant aliquots from each of the cultured fractions were then tested in parallel for either their capacity to lyse ^{51}Cr-labeled P815X2 cells (Fig. 5 *b*) or to inhibit macrophage migration in the presence of specific antigen (Fig. 5 *c*). It can be seen that while the sedimentation profiles for the precursors of these two effector activities were rather similar, they however were not coincident. The cytotoxicity profile showed peak activity generated from cells sedimenting at about 3.2 mm/h, while the activity profile for migration inhibition showed maximum activity generated not in this fraction but from slightly larger cells. If the precursors of the cells active in cytotoxicity and migration inhibition were identical then one might have expected the same fraction to generate the highest level of activity in each of the assays. This has not been the case; the finding of nonidentical fractions generating peak activity in the two assays has been a reproducible one. The results shown in Fig. 5 are therefore consistent with the hypothesis that cytotoxic and migration inhibition activities are generated from different precursor populations.

DISCUSSION

The cell-mediated immune response of mice after injection of allogeneic cells is characterized by the development of activated T cells specifically reactive toward those alloantigens. This T-cell activation can be measured in a variety of assay systems. One of the most extensively used assays has been the measurement of an activated population's capacity to specifically lyse homologous alloantigen-bearing cells in vitro. Another activity which can be quantitated is the capacity of immune cells in the presence of specific antigen to inhibit the in vitro migration of normal macrophages. The present study has investigated some of the physical and biological properties of the cells involved in mediating these two effector activities. The results of this investigation indicate that these two effector activities are indeed mediated by T cells, and that there are several experimental situations in which cytotoxic and macrophage migration inhibition activities are to a large degree dissociable, suggesting that different cells mediate these activities.

The first indication that cytotoxicity and migration inhibition were not invariably associated with each other came from the studies of the tissue distributions of these activities in animals immunized i.p. with the tumor allograft. Early studies by Brunner et al (26) had indicated that at the peak of the cellular immune response to such an i.p. allograft (approximately 2 wk), high

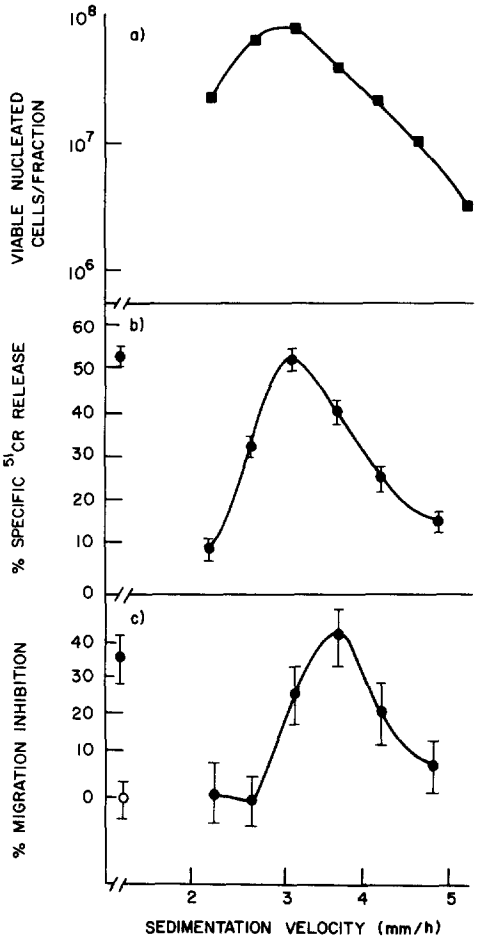

FIG. 5. Velocity sedimentation profile of the progenitors from normal C57BL spleens of cells active in cytotoxicity and macrophage migration inhibition following in vitro sensitization to alloantigen. Panel *a* shows the viable nucleated cell profile for normal C57BL spleen cells after sedimentation for 4 h at 4°C. Each fraction, as well as an aliquot of unfractionated cells, was placed in culture in the presence of irradiated BALB/c spleen cells under conditions described in detail in Materials and Methods. After 5 days the multiple cultures of each fraction were harvested and pooled, and then each fraction was resuspended to an equal volume. The fractions were then each tested in parallel for both their capacity to lyse ^{51}Cr-labeled target cells and to inhibit macrophage migration in the presence of appropriate alloantigen. The same percentage of the cells from each fraction was analyzed in either of the in vitro assays. Panel *b* shows the activity profile of progenitors of cytotoxic cells. Data points are means (±1 SEM) of triplicate determinations. Each well contained 5×10^4 target cells and 6% of the cells in a given fraction. Panel *c* shows the activity profile of progenitors of cells active in inhibiting macrophage migration. Data points are means (±95% confidence limits) of quadruplicate determinations. Each capillary tube contained 10^6 macrophages, 4×10^5 irradiated BALB/c spleen cells and 1% of the cells from a given fraction. Data points at far left of panel show migration inhibition seen with sensitized unfractionated cells in the presence of either irradiated BALB/c spleen cells (closed circle) or irradiated C57BL spleen cells (open circle).

levels of cytotoxicity were demonstrable in PBL, spleen, and MLN cell suspensions; these observations were confirmed in the present study. In addition, however, we observed that the peripheral, presumably nondraining lymph nodes from these mice displayed very little cytotoxic activity. This lack of cytotoxic activity could not readily be explained by the presence of an overriding concurrent suppressive activity in these suspensions. That PLN indeed contained substantial numbers of precursors of cytotoxic cells was demonstrated in the experiment where following subcutaneous immunization with the tumor allograft very respectable levels of cytotoxic activity were found in PLN suspensions. In this case, however, the MLN suspension (which were now presumably the distal nondraining nodes) were devoid of substantial cytotoxic activity. With either i.p. or subcutaneous immunization, PBL suspensions were cytolytic, suggesting that cytotoxic T cells could enter the blood but once having gotten there were somehow deficient in their capacity to traverse the postcapillary venular endothelium and enter or re-enter lymph node tissue (31).

On the other hand, when the PLN cells from i.p. immunized mice were tested for their capacity to specifically inhibit migration of normal mouse peritoneal macrophages they were found to be at least as active as the spleen cells from these same animals. This dissociation first suggested that distinct subpopulations of T cells could be mediating cytotoxicity and migration inhibition.

The adoptive transfer of immune spleen cells into lethally irradiated recipients provided additional support for the above hypothesis. Upon adoptive transfer of such cells into irradiated syngeneic recipients, significantly more macrophage migration inhibition activity (on a cell for cell basis) was found 24 h later in the lymph node-seeking population than in the spleen-seeking population. On the other hand cytotoxic activity was much more evident in recipient spleen cell suspensions than in PLN suspensions. It is generally accepted that one of the characteristics of recirculating lymphocytes is their capacity to migrate to lymph nodes in such adoptive transfer experiments (37, 48). Recent studies have pointed out that antigen can cause a transient nonspecific trapping of recirculating lymphocytes at the site of antigen localization (49–51). It is possible that the immune spleen cell suspension contained significant amounts of alloantigen, and that upon i.v. transfer of these suspensions such an antigen trap was operative in the recipient spleen. It could be argued that this was at least a partial explanation for the localization of cytotoxic activity in the spleen; nevertheless, this still would not explain the disparate patterns of localization for cytotoxic activity on the one hand and migration inhibition activity on the other.

The third series of experiments which support the possibility that distinct subpopulations of T cells are involved in cytotoxicity and migration inhibition were those employing 1 *g* velocity sedimentation to fractionate immune cells immediately before testing for these effector activities. Essentially similar results were obtained with either immune spleen or PLN cells. The majority

of cytotoxic activity was found with cells sedimenting at about 4 mm/h or less; on the other hand the majority of macrophage migration inhibition activity was observed with cells sedimenting at more than 4 mm/h.

Several points concerning this segregation of activities warrant comment. First of all, the separation of cytotoxic and migration inhibition activities was not absolute; though the majority of cytotoxic activity was present in smaller cells, fractions enriched for larger cells were not devoid of this activity. Indeed, on a cell for cell basis the large lymphocytes are more active than the smaller lymphocytes, but because such large cells make up only a small percentage of such immune populations their contribution to the total cytotoxic activity is rather meager. Furthermore if one looks at the activity profiles of velocity sedimented cells obtained either from spleens 8 days after in vivo immunization (instead of 14 days) or 5 days after in vitro sensitization these profiles are definitely shifted toward larger cells (30, and R. Tigelaar, unpublished observations).

While very substantial amounts of cytotoxic activity were always seen in small lymphocyte-enriched fractions, virtually no migration inhibition activity was ever seen when such cells were tested immediately after sedimentation, suggesting a rather complete separation of activities in these fractions. More recent experiments, however, which will be the subject of a separate communication, have indicated that the lack of migration inhibition activity in these fractions also is not absolute, since appropriate manipulation of this population (such as either overnight incubation, mild trypsinization, or adoptive transfer into irradiated recipients) leads to significant activity in small lymphocyte-enriched fractions when they were subsequently retested.

We have demonstrated a gross separation of T-cell-mediated alloantigen-directed cytotoxicity from macrophage migration inhibition in three types of experiments, and in so doing add to the ever-growing list of murine T-cell functions which can be dissociated from each other (52–62). These dissociations can be explained by the existence of distinct subpopulations of T cells with discrete and limited functional capabilities. Since none of the manipulations used in these experiments resulted in populations completely devoid of either cytotoxic or migration inhibition activity, the present experiments do not permit a categorical statement that a given subpopulation of T cells has only one or the other of these particular effector functions. While this has certainly not been excluded, several other possibilities come to mind. Some activated T cells may express both functions and others only one. It is also possible that during antigen-stimulated proliferations and differentiation, expression of a given effector activity is a function of the cell cycle: i.e., at a given point in the cell cycle one effector activity is either preferentially expressed (or preferentially suppressed or rendered capable of being suppressed) in relation to another activity. For example, certain cultured cell lines have been shown to selectively release a macrophage migration inhibitory factor during S phase (63).

The relationship of the cells involved in the functions studied here to those in other assays of T-cell responsiveness has not been fully investigated. It is worth reiterating the observations of Grant et al (61), who noted no direct cytotoxicity but substantial antigen-specific macrophage arming activity in suspensions of thymocytes from i.p. tumor allografted mice, although both activities were present in spleen suspensions from these mice. This appears to be rather similar to the dissociation we observed between immune spleen cells (good activity in both assays) and immune PLN cells (good activity in migration inhibition assay, little activity in cytotoxicity assay). Dr. G. M. Iverson, utilizing the identical experimental model, has observed that on a cell for cell basis immune PLN cells are as active as immune spleen cells in their capacity to release specific macrophage arming factor (SMAF) (personal communication); it is very possible that the same cell type is responsible for SMAF release and inhibiting macrophage migration.

It is probable that with regard to effector functions a single antigen-activated T cell has a limited repertoire and perhaps is even restricted to playing a single tune. A crucial question is at what point does this commitment take place? In an attempt to approach this problem we asked the perhaps naive question of whether there was any difference between the velocity sedimentation profile of the splenic precursors of cytotoxic lymphocytes and the profile of the splenic precursors of cells capable of inhibiting macrophage migration. The experiments represented in Fig. 5 would indicate that there may be such a difference. The fraction of normal spleen cells which after in vitro sensitization showed the most cytotoxic activity did not exhibit the maximum migration inhibition activity. If there was a common splenic precursor cell for these effector activities, one might have expected the activity profiles to be coincident. Thus it is possible that in the spleen of a nondeliberately alloantigen-stimulated adult animal commitment to either of these particular effector functions has already taken place. However it must also be considered that this commitment may not occur until a common multipotential splenic precursor cell confronts antigen, and that the particular effector activity which this cell can express is determined by the precise conditions under which the antigen is confronted. This in turn could be influenced by a number of other factors including the presence or quantity of any other cell or cells which could exert some type of regulatory influence on the differentiating T cell.

SUMMARY

The immune response of C57BL mice to a DBA/2 tumor allograft has been assessed in two assays of cell-mediated immunity, the in vitro lysis of ^{51}Cr-labeled target cells and the antigen-mediated inhibition of macrophage migration. Both assays were shown to be measuring a T-cell-mediated reaction. Three types of experiments suggested that distinct subpopulations of T cells mediate these reactions. The tissue distributions of these activities was dis-

tinctive; both activities were present in spleens from i.p. immunized mice, but only macrophage migration inhibition activity was found in the peripheral lymph nodes (PLN) of such mice. Adoptive transfer of immune spleen cells into irradiated syngeneic recipients revealed that while a substantial amount of migration inhibition activity could subsequently be found in PLN, cytotoxic activity was found predominantly in the spleens of these adoptive hosts. Velocity sedimentation analysis of immune cells 14 days after i.p. immunization indicated that while the majority of cytotoxic activity was associated with small and medium lymphocytes, the majority of migration inhibition activity was associated with medium and large lymphocytes. In addition, normal spleen cells were fractionated by velocity sedimentation immediately before allosensitization in vitro. Subsequent analysis of the sensitized fractions revealed that the activity profiles for cytotoxicity and macrophage migration inhibition were not coincident. The implications of these observations are discussed.

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REFERENCES

1. Warner, N. L., A. Szenberg, and F. M. Burnet. 1962. The immunologic role of different lymphoid organs in the chicken. I. Dissociation of immunological responsiveness. *Aust. J. Exp. Biol. Med. Sci.* **40**:373.
2. Good, R. A., and A. E. Gabrielsen. 1968. The thymus and other lymphoid organs in the development of the immune system. *In* Human Transplantation. F. Rapaport and J. Dausset, editors. Grune and Stratton, Inc., New York. 526.
3. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen reactive cells. *Transplant. Rev.* **1**:3.
4. Davies, A. J. S. 1969. The thymus and the cellular basis of immunity. *Transplant. Rev.* **1**:43.
5. Claman, N. H., and E. A. Chaperon. 1969. Immunologic complementation between thymus and marrow cells—a model for the two-cell theory of immunocompetence. *Transplant. Rev.* **1**:92.
6. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody producing cells. *Science* (*Wash. D. C.*). **140**:405.
7. Cunningham, A. J. 1965. A method of increased sensitivity for detecting single antibody forming cells. *Nature* (*Lond.*). **207**:1106.
8. Nossal, G. J. V., A. E. Bussard, H. Lewis, and J. C. Mazie. 1970. In vitro stimulation of antibody formation by peritoneal cells. I. Plaque technique of high sensitivity enabling access to the cells. *J. Exp. Med.* **131**:894.
9. Nossal, G. J. V., H. Lewis, and N. L. Warner. 1971. Differential sensitivity of haemolytic plaque methods at various stages of the immune response. *Cell. Immunol.* **2**:13.
10. Nossal, G. J. V., N. L. Warner, and H. Lewis. 1971. Incidence of cells simultaneously secreting IgM and IgG antibody to sheep erythrocytes. *Cell. Immunol.* **2**:41.

11. Nordin, A. A., H. Cosenza, and S. Sell. 1970. Immunoglobulin class of antibody-forming cells in mice. II. Class restriction of plaque-forming cells demonstrated by replica plating. *J. Immunol.* **104:**495.
12. Merchant, B., and Z. Brahmi. 1970. Duplicate plating of immune cells products: analysis of globulin class secretion by single cells. *Science (Wash. D. C.).* **167:**69.
13. Miller, H. C., and G. Cudkowicz. 1971. Density gradient separation of marrow cells restricted for antibody class. *Science (Wash. D. C.).* **171:**913.
14. Mäkelä, O., and A. M. Cross. 1970. The diversity and specialization of immunocytes. *Prog. Allergy.* **14:**145.
15. Boyle, W. 1968. An extension of the ^{51}Cr-release assay for the estimation of mouse cytotoxins. *Transplantation.* **6:**761.
16. Gorczynski, R. M., R. G. Miller, and R. A. Phillips. 1973. Reconstitution of T cell-depleted spleen cell populations by factors derived from T cells. I. Conditions for the production of active T cell supernatants. *J. Immunol.* **110:**968.
17. Golub, E. 1971. Brain-associated θ antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. *Cell. Immunol.* **2:**353.
18. Gorczynski, R. M. 1974. Evidence for *in vivo* protection against murine-sarcoma virus-induced tumors by T lymphocytes from immune animals. *J. Immunol.* **112:**533.
19. Raff, M. C. 1970. Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology.* **19:**637.
20. Greaves, M. F., and G. Janossy. 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. *Transplant. Rev.* **11:**87.
21. Feldmann, M., and A. J. Basten. 1971. The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. *J. Exp. Med.* **134:**103.
22. Miller, R. G., and R. A. Phillips. 1969. Separation of cells by velocity sedimentation. *J. Cell. Physiol.* **73:**191.
23. Miller, R. G., and R. A. Phillips. 1970. Sedimentation analysis of the cells in mice required to initiate an *in vivo* immune response to sheep erythrocytes. *Proc. Soc. Exp. Biol. Med.* **135:**63.
24. Dunn, T. B., and M. Potter. 1957. A transplantable mast-cell neoplasm in the mouse. *J. Natl. Cancer Inst.* **18:**587.
25. Gorer, P. A. 1956. Some recent work on tumor immunity. *Adv. Cancer Res.* **4:**149.
26. Brunner, K. T., J. Mauel, H. Rudolf, and B. Chapius. 1970. Studies of allograft immunity in mice. I. Induction, development and *in vitro* assay of cellular immunity. *Immunology.* **18:**501.
27. David, J. R., S. Al-Askari, H. S. Lawrence, and J. L. Thomas. 1964. Delayed hypersensitivity *in vitro*. I. The specificity of inhibition of cell migration by antigens. *J. Immunol.* **93:**264.
28. Canty, T., J. R. Wunderlich, and F. Fletcher. 1971. Qualitative and quantitative studies of cytotoxic immune cells. *J. Immunol.* **106:**200.
29. Wagner, H., and M. Feldmann. 1972. Cell-mediated immune response *in vitro*. I. A new system for the generation of cell-mediated cytotoxic activity. *Cell. Immunol.* **3:**405.
30. MacDonald, H. R., R. A. Phillips, and R. G. Miller. 1973. Allograft immunity in the mouse. I. Quantitation and specificity of cytotoxic effector cells after *in vitro* sensitization. *J. Immunol.* **111:**565.

31. Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. R. Soc. Lond. B. Biol. Sci.* **159**:257.
32. Canty, T. G., and J. R. Wunderlich. 1970. Quantitative *in vitro* assay of cellular cytotoxic immunity. *J. Natl. Cancer Inst.* **45**:761.
33. Brunner, K. T., and J. C. Cerottini. 1971. Cytotoxic lymphocytes as effector cells of cell-mediated immunity. *In* Progress in Immunology. B. Amos, editor. Academic Press, Inc., New York. 385.
34. Häyry, P. L., L. C. Andersson, S. Nordling, and M. Virolainen. 1972. Allograft response *in vitro*. *Transplant. Rev.* **12**:91.
35. Gorczynski, R. M. 1974. Immunity to murine-sarcoma virus-induced tumors. I. Specific T lymphocytes active in macrophage migration inhibition and lymphocyte transformation. *J. Immunol.* **112**:1815.
36. Gorczynski, R. M. 1974. Immunity to murine-sarcoma virus-induced tumors. II. Suppression of T cell-mediated immunity by cells from progressor animals. *J. Immunol.* **112**:1826.
37. Lance, E. M., and Taub, R. N. 1969. Segregation of lymphocyte populations through differential migration. *Nature* (*Lond.*). **221**:841.
38. Morse, H. C., III, and R. Asofsky. 1974. *In vivo* effects of antithymocyte serum on the homing patterns and graft-*versus*-host reactivity of murine splenic lymphocytes. *Cell. Immunol.* **11**:13.
39. Cerottini, J. C., A. A. Nordin, and K. T. Brunner. 1970. *In vitro* cytotoxicity of thymus cells sensitized to alloantigens. *Nature* (*Lond.*). **227**:72.
40. Wagner, H., A. W. Harris, and M. Feldmann. 1972. Cell-mediated immune response *in vitro*. II. The role of thymus and thymus-derived lymphocytes. *Cell. Immunol.* **4**:39.
41. Evans, R., and P. Alexander. 1972. Mechanism of immunologically specific killing of tumor cells by macrophages. *Nature* (*Lond.*). **236**:170.
42. Evans, R., and P. Alexander. 1972. Role of macrophages in tumor immunity. III Cooperation between macrophages and lymphoid factors in an *in vitro* allograft situation. *Immunology*. **23**:677.
43. Lohmann-Matthes, M. D., H. Schipper, and H. Fischer. 1972. Macrophage-mediated cytotoxicity against allogeneic target cells *in vitro*. *Eur. J. Immunol.* **2**:45.
44. MacLennon, I. C. M., G. Loewi, and A. Howard. 1969. A human serum immunoglobulin with specificity for certain homologous target cells, which induces target cell damage by normal human lymphocytes. *Immunology*. **17**:897.
45. Rosenstreich, D. L., J. T. Blake, and A. S. Rosenthal. 1971. The peritoneal exudate lymphocyte. I. Differences in antigen responsiveness between peritoneal exudate and lymph node lymphocytes from immunized guinea pigs. *J. Exp. Med.* **134**:1170.
46. Rosenthal, A. S., J. M. Davie, D. L. Rosenstreich, and J. T. Blake. 1972. Depletion of antibody-forming cells and their precursors from complex lymphoid cell populations. *J. Immunol.* **108**:279.
47. Yoshida, T., H. Souozaki, and S. Cohen. 1973. The production of migration inhibition factor by B and T cells of the guinea pig. *J. Exp. Med.* **138**:784.
48. Taub, R. N., and E. M. Lance. 1971. Effects of lymphoic depletion on the distribution of ^{51}Cr-labelled lymph node cells in mice. *Transplantation*. **11**:536.

49. Zatz, M., and E. M. Lance. 1971. The distribution of ^{51}Cr-labelled lymphocytes into antigen-stimulated mice. Lymphocyte trapping. *J. Exp. Med.* **134**:224.
50. Zatz, M., and E. M. Lance. 1971. Lymphocyte trapping in tolerant mice. *Nat. New Biol.* **234**:253.
51. Frost, P., and E. M. Lance. 1973. Abrogation of lymphocyte trapping by ascitic tumours. *Nature (Lond.)*. **246**:101.
52. Asofsky, R., R. Tigelaar, and H. Cantor. 1971. Cell interactions in the graft-*versus*-host response. *In* Progress in Immunology. B. Amos, editor. Academic Press, Inc., New York. 369.
53. Cantor, H., and R. Asofsky. 1972. Synergy among lymphoid cells mediating the graft-*vs*.-host response. III. Evidence for interaction between two types of thymus-derived cells. *J. Exp. Med.* **135**:764.
54. Tigelaar, R. E., and R. Asofsky. 1973. Synergy among lymphoid cells mediating the graft-*vs*.-host response. V. Derivation by migration in lethally irradiated recipients of two interacting subpopulations of thymus-derived cells from normal spleen. *J. Exp. Med.* **137**:239.
55. Cohen, L., and M. L. Howe. 1973. Synergism between subpopulations of thymus-derived cells mediating the proliferative and effector phases of the mixed lymphocyte reaction. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2707.
56. Wagner, H. 1973. Synergy during in vitro cytotoxic allograft responses. I. Evidence for cell interaction between thymocytes and peripheral T cells. *J. Exp. Med.* **138**:1379.
57. Tigelaar, R. E., and M. Feldmann. 1973. Synergy among thymocytes and peripheral lymph node cells in the *in vitro* generation of lymphocytes cytotoxic to alloantigens. *Transplant. Proc.* **5**:1711.
58. Kano, S., B. R. Bloom, and M. L. Howe. 1973. Enumeration of activated thymus-derived lymphocytes by the virus plaque assay. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2299.
59. Stobo, J. D., A. S. Rosenthal, and W. E. Paul. 1972. Functional heterogeneity of murine lymphoid cells. I. Responsiveness to and surface binding of concanavalin A and phytohemagglutinin. *J. Immunol.* **108**:1.
60. Stobo, J. D., and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. III. Differential responsiveness of T cells to phytohemagglutinin and concanavalin A as a probe for T cell subsets. *J. Immunol.* **110**:362.
61. Grant, C. K., G. A. Currie, and P. Alexander. 1972. Thymocytes from mice immunized against an allograft render bone-marrow cells specifically cytotoxic. *J. Exp. Med.* **135**:150.
62. Gordon, J., and H. Yu. 1973. Relationship of T cells involved in cell mediated immunity and antibody synthesis. *Nat. New Biol.* **244**:21.
63. Tubergen, D. G., J. D. Feldman, E. M. Pollack, and R. A. Lerner. 1972. Production of macrophage migration inhibition factor by continuous cell lines. *J. Exp. Med.* **135**:255.