Short paper

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Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage*

Previous reconstitution studies in irradiated mice distinguished Ly-1 B from other B cells. The predominant B cell populations in spleen and lymph node are readily reconstituted from progenitors present in adult bone marrow. Ly-1 B cells, in contrast, are reconstituted from cells in peritoneum rather than bone marrow. Further studies, presented here, demonstrate that Ly-1 B populations are reconstituted from peritoneal cells that already express both surface immunoglobulin (IgM) and Ly-1, that is, Ly-1 B. Such cells reconstitute and maintain the Ly-1 B population in irradiated recipients for at least 6 months.

1 Introduction

Ly-1 bearing B cells (Ly-1 B) express the Ly-1 surface glycoprotein in conjunction with characteristic amounts of classical B cell surface molecules such as IgM, IgD, and Ia. These unique lymphocytes are rare (1–2%) in spleen and undetectable in lymph nodes and Peyer's patches [1, 2]. In contrast to this low frequency in adult lymphoid tissues, Ly-1 B constitute a large fraction of surface IgM^+ B cells in early development: almost one-third of surface IgM^+ B cells in neonatal mice are Ly-1 B [1–3]. Curiously, whereas the relative frequency of Ly-1 B rapidly decreases as mice age, these cells constitute a large proportion of the B cells found in peritoneum even in the adult [2, 6]. In addition, their representation is increased among B cells from both spleen and peritoneum of certain autoimmune strains, *e.g.* NZB [1] and Motheaten mice [4]. This increase appears to be related to the observation that Ly-1 B are capable of producing most of the commonly studied murine IgM autoantibodies [5]. One autoantibody in particular, found in most strains and reactive with bromelain-treated mouse red blood cells, is closely correlated with the level of Ly-1 B [2, 5].

In a previous report [6] we showed that Ly-1 B, distinct from other B cells by a variety of criteria, constitute a distinct developmental lineage. These experiments were based on protocols in which cells from various progenitor sources were transferred into lethally irradiated allotype (Igh)-congenic recipients. The resultant B cell populations were characterized both by fluorescence-activated cell sorter (FACS) and functional analyses 1 to 6 months later using monoclonal antibodies specific for immunoglobulin allotype.

Results from these earlier studies established (a) that liver and spleen from mice less than 1 week old or bone marrow (BM) from donors under 6 weeks of age reconstitute all B cells; and (b) that progenitor activity segregates in adults such that BM selectively reconstitutes the predominant splenic or lymph node B cell populations while peritoneal cells (PerC) selectively reconstitute Ly-1 B. Although we have found that the lack of Ly-1 B progenitors in adult BM is not absolute (since Ly-1 B generation from adult BM has occasionally been observed in a subsequent transfer experiment [7]), the finding of segregated B cell generation strongly suggests that the progenitors for Ly-1 B are independently maintained from those for other B cells.

We report here on studies of the cells responsible for reconstituting Ly-1 B. We show that the cells capable of generating and maintaining Ly-1 B from adult animals are surface Ig^+ Ly-1 B and that surface Ig^- cells from adult peritoneum lack B cell precursor activity. In addition, we find that surface Ig^- precursors for Ly-1 B do exist in spleens from young mice.

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Abbreviations: **Ly-1 B:** Ly-1 bearing B cells **FACS:** Fluorescence-activated cell sorter **BM:** Bone marrow **PerC:** Peritoneal washout cells

2 Materials and methods

2.1 Mice

CBA mice congenic for Ig allotype (CBA-Igh^b) were established by Dr. H. S. Micklem (University of Edinburgh, Scotland) and were his generous gift. CBA/N mice were also obtained from Dr. Micklem and bred in our animal facility. BALB/cN and BAB/25 mice were raised in our colony.

2.2 Radiation and cell transfer

Four to 6-month-old recipients were irradiated with 750 rds (for CBA or CBA/N) or 600 rds (for BALB/c) by X-ray 1 day before cell transfer. Femur and tibia of 3–5 month-old (adult) mice were used as sources of adult BM cells. Per C were harvested from adult mice by injecting chilled medium as described previously [6]. The irradiated mice received $5 \times 10^6 - 10 \times 10^6$ cells, usually injected i.v. For FACS sorting experiments, cells corresponding to $5 \times 10^6 - 10 \times 10^6$ unseparated cells (either adult PerC or neonatal spleen) from Igh-6b mice were sorted and transferred together with $1 \times 10^6 - 5 \times 10^6$ BM cells from adult mice of a different allotype.

2.3 Cell staining and antibodies

Peritoneal cells and spleen cells from transferred mice were examined for reconstitution at the various times specified. Reconstitution of donor (Igh^b)-derived B cells was determined by immunofluorescence staining with monoclonal anti-Ig allotype reagents, anti-Igh-6b (IgM^b, AF6-78.25) [8] and anti-Igh-5b (IgD^b, AF6-122.2) [8]. The level of either donor- or

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recipient-derived B cell reconstitution was estimated by comparison of total B cells (stained with rat anti-mouse IgM, 331.12) [9] with anti-Igh-6b$^+$ B cells. The frequency of Ly-1 B was obtained from two-color staining of cells with fluorescein (Fl)-anti-IgM (or Igh-6b) und bimtinated (Bi)-anti-Ly-1 antibody (53-7.3) [10] together with Texas Red (TR)-avidin. Anti-Lyt-2 antibody (53-6.7) [10], which is the same rat isotype as anti-Ly-1, was used as a control in staining for Ly-1 B. Both anti-Ly-1 and anti-Lyt-2 antibodies were employed as F(ab')$_2$ reagents. Light chain expression on B cells was determined by using rat monoclonal antibody, anti-kappa (187.1) [11] and goat anti-mouse lambda antibody kindly provided by Dr. A. Radbruch, Univ. of Köln [2]. Preparation of cells, detailed staining procedure, purification of rat monoclonal antibodies, Fl, TR and biotin (Bi) labeling, and preparation of rat F(ab')$_2$ antibodies have been described in previous reports [1, 5, 6].

2.4 Staining data analysis

Two-color staining data were analyzed with a modified FACS (FACS II; Becton Dickinson Immunocytometry Systems, Inc., Mountain View, CA) and VAX computer system. Only cells with forward and large angle scatter typical of lymphocytes are presented in the figures showing peritoneal reconstitution. In particular, granular cells that scatter large amounts of light to the photodetector measuring light at large angles are eliminated from these analyses [2, 6]. Tabulated frequencies, however, represent percent of total recovered cells (including granular cells).

3 Results

3.1 Ly-1 B progenitors in PerC are contained within the FACS-sorted Ly-1 B cell population

The experimental protocols used here are similar to those used in previous studies [6]. Peritoneal cells of Igh^b allotype mice (CBA-Igh^b) were transferred into lethally irradiated allotype-congenic recipients (CBA/N or CBA) and the resultant B cell populations were characterized by FACS 1 to 6 months later. Adult BM from mice with the same allotype as recipient (CBA) was used to supplement other hemopoietic cells. Most of the B cells in these recipients derive from traditional B cell progenitors in the BM and thus do not express the Igh^b allotype. The Ly-1 B, however, tend to be derived from peritoneal progenitors and therefore mainly express the Igh^b allotype of the PerC donor, as clearly shown in PerC analysis (Fig. 1, left panels).

Using FACS-sorted Ly-1 B from the peritoneum in place of total peritoneal cells in these kinds of transfers yields similar results. That is, Ly-1 B levels reached in irradiated recipients of FACS-sorted IgM$^+$, Ly-1$^+$ (Ly-1 B) cells supplemented with recipient-type BM are equivalent to the Ly-1 B levels reached in animals restored with unsorted PerC (similarly supplemented). Thus, FACS-sorted Ly-1 B from PerC contain progenitors that fully and permanently reconstitute Ly-1 B in irradiated recipients (Fig. 1, middle panels).

FACS-sorted IgM$^-$ cells from PerC, in contrast, have no detectable B cell progenitor activity. In general, co-transferring this Ig-depleted population together with recipient-type BM is equivalent to transferring recipient-type BM alone. All recipients develop fully reconstituted BM-derived B cell populations and nearly all lack substantial numbers of Ly-1 B cells. When detectable, Ly-1 B in these recipients (CBA) tend to be most numerous several months after transfer and to express the recipient allotype (data not shown). These cells may be derived either from host cells that survive irradiation (Ly-1 B are relatively radioresistant; [6]) or from progenitors that are sporadically found in BM [7]. Favoring the former possibility, CBA/N mice that normally lack peritoneal Ly-1 B never develop this recipient allotype Ly-1 B population in such transfer experiments. In any event, the immunoglobulin allotypes they express show clearly that they are not derived from the IgM$^-$ peritoneal cell population.

Six months after transfer, recipients of FACS-purified Ly-1 B cells (like recipients of unsorted PerC) still have normal numbers of PerC-derived Ly-1 B (10–25%). Furthermore, recipients of FACS-purified Ig$^-$ cells still lack PerC-derived Ly-1 B (<1%). Thus, PerC contains self-renewing progenitors that reconstitute and maintain the Ly-1 B population in irradiated recipients and most or all of these progenitors are present in the PerC Ly-1 B cell population itself, *i.e.*, in the FACS-sorted IgM$^+$, Ly-1$^+$ population from PerC.

3.2 Progenitors that reconstitute peritoneal Ly-1 B cells also reconstitute splenic Ly-1 B cells

There are normally fewer Ly-1 B cells in spleen than in peritoneum; however, the splenic Ly-1 B population is clearly detectable as a small group of cells with the typical Ly-1 B surface phenotype (bright IgM, dull IgD and dull Ly-1) in adult PerC transferred mice. Like the peritoneal Ly-1 B population, this splenic Ly-1 B population is permanently reconstituted in recipients of FACS-sorted peritoneal Ly-1 B cells (Fig. 2) but not in recipients of FACS-sorted Ig$^-$ peritoneal cells.

3.3 Neonatal spleen contains Ig$^-$ progenitors for Ly-1 B

We have previously shown that in contrast to adult mice, spleen and liver from neonatal mice are capable of reconstituting Ly-1 B [5]. Ly-1 B are, in fact, a major population among surface IgM$^+$ cells in neonatal mice. We next attempted to examine whether such self-maintaing ability is also the case for Ly-1 B present in early development.

FACS-sorted Ig$^+$ spleen cells from 2–7-day-old animals reconstitute mostly Ly-1 B cells when transferred to allotype congenic recipients (lethally irradiated and supplemented with syngeneic BM as in the preceeding experiments). The reconstitution per IgM$^+$ cell transferred is less efficient than the reconstitution obtained by transferring IgM$^+$ adult PerC: however, this relatively small Ly-1 B population contains typical Ly-1 B (Fig. 3, left) and persists for at least 6 months in recipients.

FACS-sorted Ig$^-$ cells from neonatal spleen also contain progenitors that reconstitute Ly-1 B (Fig. 3, right). Since adult spleen does not repopulate Ly-1 B [6], these progenitors apparently represent earlier Ly-1 B differentiation stage(s) that tend to disappear or to become less functional with respect to giving rise to Ly-1 B as the animal ages. As such, they represent a potential source for the wide variety of pre-B cell tumors that carry the Ly-1 surface antigen, *e.g.* 70Z [12, 13], NFS-5 [12, 13] and a series of neonatally derived tumors induced with the Abelson virus (H. C. Morse III, personal communication).

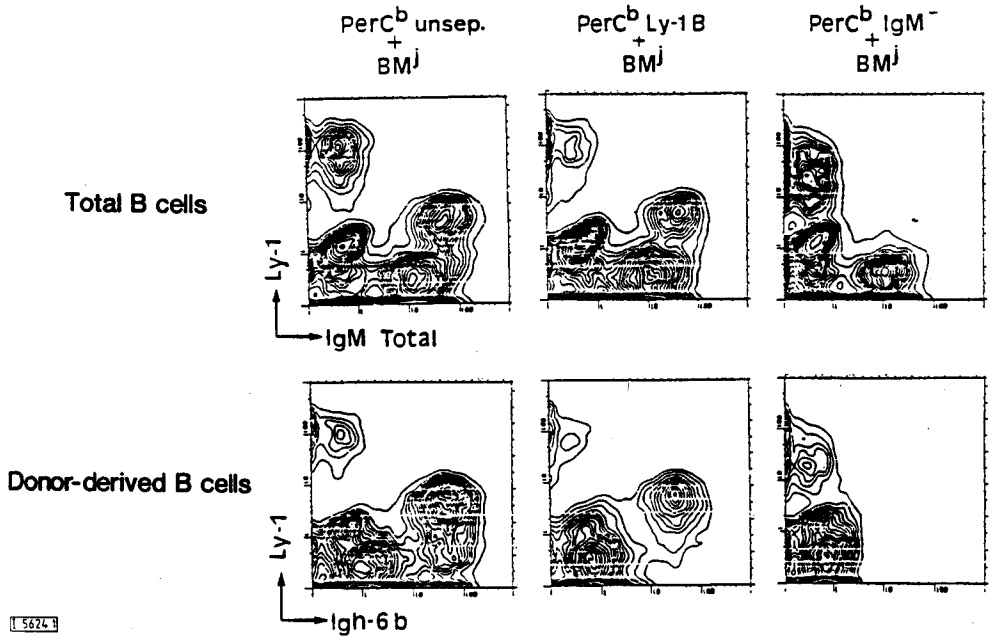


Figure 1. Reconstituted Ly-1 B in PerC are derived from Ly-1 B. Peritoneal cells from adult CBA-Igh^b mice were stained with Fl-anti-IgM and Bi-anti-Ly-1 (+ TR-avidin). Unsorted stained cells, sorted Ly-1 B (Ly-1$^+$/IgM$^+$) and sorted IgM$^-$ cells (mostly free of Ly-1 bright T cells) were injected into irradiated CBA/N mice together with 5×10^6 adult CBA BM cells. All sorted cells fell within the typical lymphocyte range for both forward and obtuse light scatter (eliminating granular cells). One $\times 10^7$ unseparated PerC cells and corresponding numbers of sorted cells (2×10^6 Ly-1 B cells, 0.5×10^6 IgM$^-$ cells) were injected per mouse. Analyses one month after transfer are presented in this figure. The reconstitution of donor allotype Ly-1 B by unseparated PerC, Ly-1 B, and IgM$^-$ cells was 14%, 10%, and < 1%, respectively (see populations in lower panels). Ly-1$^-$ B cells found almost equally (10–20%) in each group of transferred mice shown in the upper panels are predominantly BM derived (not 'b' allotype; compare with lower panels). Six months after transfer, the relative composition of PerC B cells in each reconstituted group was similar to this one month result.

The relationship of these neonatal splenic Ig$^-$ progenitors of Ly-1 B to the B cell progenitors that give rise to the predominant B cell populations in spleen and lymph nodes (Ly-1$^-$/IgD high B cells) has yet to be determined. They appear to be different since (as we have indicated) adult spleen contains ample progenitors that reconstitute the predominant B cell populations but do not detectably reconstitute Ly-1 B [6].

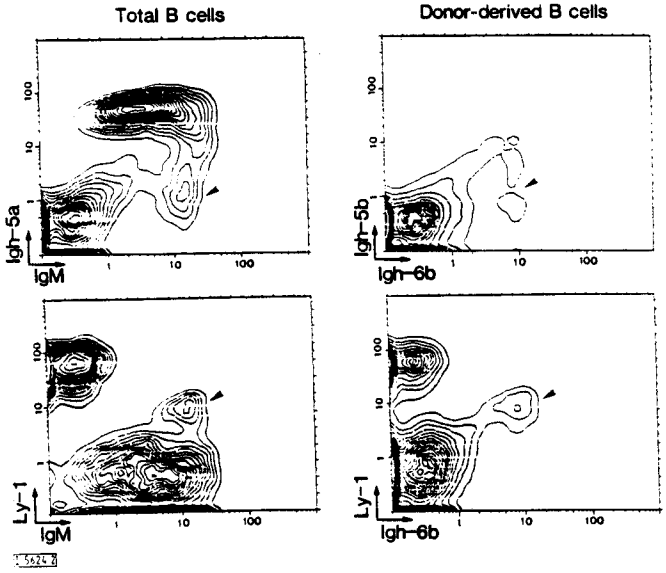


Figure 2. Stable reconstitution of Ly-1 B in spleen by sorted PerC Ly-1 B. Spleen cells from CBA/N mice that had been irradiated and transferred with a mixture of sorted CBA-Igh^b PerC Ly-1 B together with adult CBA BM were stained for IgM/IgD and IgM/Ly-1 expression 6 months after transfer. In addition to large numbers of BM-derived Ly-1$^-$ B cells, Ly-1 B found in these reconstituted mice were all 'b' allotype with a frequency (3%) in the range found one month after reconstitution with unseparated PerC, implying that transferred Ly-1 B are maintained stably in both spleen and peritoneum. This long-term Ly-1 B population is marked in the figure.

3.4 Ig light chains (κ or λ) are present on Ly-1 B progenitors in adult PerC

Studies with a Ly-1 bearing tumor cell line (NFS-5) that differentiates *in vitro* from Ig$^-$ to Ig$^+$ with continuous expression of Ly-1 reveal a stable differentiation stage in which cells express surface μ (IgM) heavy chains without associated light chains ([12] and manuscript in preparation by Hardy et al.). The existence of this stage during the differentiation of the tumor raises the possibility that the normal Ly-1 B development

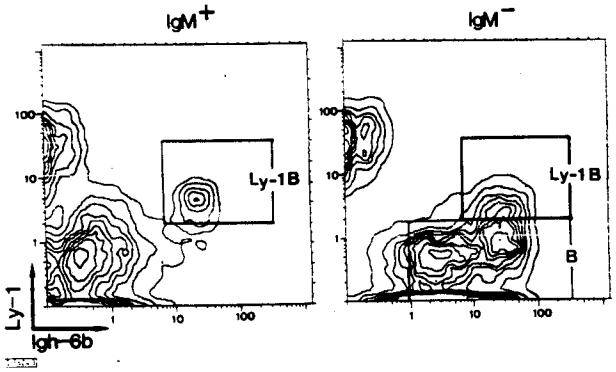
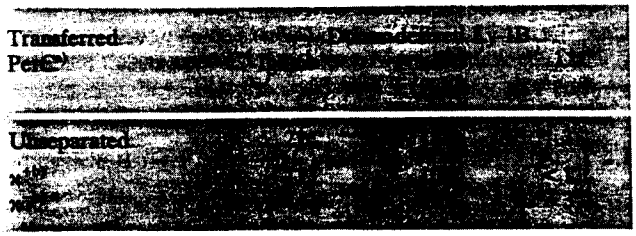


Figure 3. Neonatal spleen contains surface Ig$^+$ and Ig$^-$ progenitors for Ly-1 B. Spleen cells pooled from three 3-6-day-old BAB/25 mice were stained with Fl-anti-IgM and sorted into surface Ig$^+$ and Ig$^-$ fractions. Five $\times 10^5$ IgM$^+$ cells or 5×10^5 IgM$^-$ cells were injected into BALB/c mice together with 2×10^6 BALB/c BM. Three months later, PerC B cells derived from transferred neonatal spleen were determined by staining with Fl-anti-Igh-6b and Bi-anti-Ly-1 plus TR-avidin. The regions used for the integration of Ly-1 B and B (Ly-1$^-$) are boxed. Ly-1 B generated by the transfer of IgM$^+$ cells and IgM$^-$ cells are 6% and 10%, respectively. In addition to Ly-1 B, sorted surface IgM$^-$ cells yielded 60% typical Ly-1$^-$ B cells (which mostly express a high level of IgD). There were 50% BALB/c BM-derived B cells in the IgM$^+$ cell transferred mice; in contrast, most B cells reconstituted in the IgM$^-$ cell transferred mice were Igh-6b$^+$.

pathway includes a similar developmental stage. However, this does not appear to be the case. Cells at this stage do not represent a major proportion of the sorted IgM^+ cells shown above to reconstitute Ly-1 B since virtually all Ly-1 B in peritoneum have associated light chains (approximately 80 percent κ and 20 percent λ, [2]). Furthermore, as Table 1 shows, FACS-sorted light chain-bearing cells fully reconstitute the Ly-1 B population. Thus, most (if not all) of the peritoneal cells responsible for the long-term reconstitution of Ly-1 B cells in irradiated recipients are Ly-1 B that have complete surface Ig molecules.

In addition, as Table 1 also shows, the FACS-sorted κ^+ cells selectively reconstitute κ^+ Ly-1 B populations in transfer recipients and, similarly, FACS-sorted κ^- selectively reconstitute λ^+ Ly-1 B. Thus, Ly-1 B are reconstituted by peritoneal cells carrying surface Ig molecules whose heavy and light chain composition reflects the heavy and light chain composition of the surface Ig of the reconstituted population.

Table 1. Reconstituting Ly-1 B express complete surface Ig molecules



a) PerC from BAB/25 mice were stained with Fl-anti-mouse kappa.
b) κ^+ cells (1.5×10^6) and κ^- cells (0.7×10^6, including T cells) corresponding to 5×10^6 unseparated PerC were sorted and transferred into BALB/c mice (with 1×10^6 BALB/c BM). Two months after transfer, recipient mice were killed and examined for light chain expression of reconstituted (donor-derived) PerC Ly-1 B. PerC were stained with either Fl-anti-Igh-6b, Fl-anti-kappa or Fl-anti-lambda together with Bi-anti-Ly-1 plus TR-avidin.

4 Discussion

The repopulation of lymphoid organs depleted by irradiation or drug treatment has been studied extensively for over two decades [14–17]. Not surprisingly, the consensus view of B cell development is based largely on the properties of BM-derived B cells (since these cells represent more than 95% of the total B cell population in normal adult mice). By and large, therefore, IgM-bearing lymphocytes are treated as relatively mature cells that have a limited capacity for self-renewal (except when triggered by antigen) and must continually be replenished from self-renewing Ig^- progenitors present in spleen and BM in adults [18–21]. This formulation accurately reflects the developmental behavior of the BM-derived B cell populations in mice; however, as we have shown here, rules for replenishing the murine Ly-1 B lineage are clearly different.

Reconstitution studies in birds also challenge the generality of the current B cell development paradigm. Pink and colleagues have recently shown that B cell progenitors in the bursae of young normal chickens carry surface IgM [22]. These progenitors, which fully and permanently reconstitute B cells in cyclophosphamide-treated neonatal chickens, disappear from the bursa as the birds age so that the sole source of B cells in adult birds is a pool of self-renewing, IgM-bearing B cells with the capacity to proliferate in peripheral organs [22, 23]. Thus, the surprising reconstitution capability that we have demonstrated for peritoneal Ly-1 B in mice may well reflect the isolated use in mammals of a self-renewal capability that is used generally to maintain B cell populations in birds (and perhaps other phylogenetically primitive species).

Unfortunately, this conclusion must be tempered at present by questions concerning the extent to which the transferred murine Ly-1 B actually proliferate. The (estimated) number of Ly-1 B present in the recipient 6 months after transfer is only modestly higher than the number of Ig^+ peritoneal cells transferred [7]. Thus, if Ly-1 B are extremely long-lived cells and if all transferred Ly-1 B are recovered, very little Ly-1 B proliferation would have to occur to account for the reconstitution of the recipient Ly-1 B population. Such longevity and recovery seems unlikely; however, it is not beyond the realm of possibility. Further studies are still required to ascertain whether Ly-1 B actually represent the mammalian homolog of a more primitive B cell population.

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5 References

1 Hayakawa, K., Hardy, R. R., Parks, D. R. and Herzenberg, L. A., *J. Exp. Med.* 1983. *157:* 202.
2 Hayakawa, K., Hardy, R. R. and Herzenberg, L. A., *Eur. J. Immunol.* 1986. *16:* 450.
3 Hardy, R. R. and Hayakawa, K., *Immunol. Rev.* 1986. vol. *93,* in press.
4 Sidman, C. L., Shultz, L. D., Hardy, R. R., Hayakawa, K. and Herzenberg, L. A., *Science* 1986. *232:* 1423.
5 Hayakawa, K., Hardy, R. R., Honda, M., Herzenberg, L. A., Steinberg, A. D. and Herzenberg, L. A., *Proc. Natl. Acad. Sci. USA* 1984. *81:* 2494.
6 Hayakawa, K., Hardy, R. R., Herzenberg, L. A. and Herzenberg, L. A., *J. Exp. Med.* 1985. *161:* 1554.
7 Herzenberg, L. A., Stall, A. M., Lalor, P. and Herzenberg, L. A., *Immunol. Rev.* 1986, in press.
8 Stall, A. M. and Loken, M. R., *J. Immunol.* 1984. *132:* 787.
9 Kincade, P. W., Lee, G., Sun, L. and Watanabe, T., *J. Immunol. Methods* 1981. *42:* 17.
10 Ledbetter, J. A. and Herzenberg, L. A., *Immunol. Rev.* 1979. *47:* 63.
11 Yelton, D. E., Desaymard, C. and Scharff, M. D., *Hybridoma* 1981. *1:*5.
12 Hardy, R. R., Dangl, J. L., Hayakawa, K., Jager, G., Herzenberg, L. A. and Herzenberg, L. A., *Proc. Natl. Acad. Sci. USA* 1986. *83:* 1438.
13 Hardy, R. R., Hayakawa, K., Herzenberg, L. A., Morse, H. C., III, Davidson, W. F. and Herzenberg, L. A., *Curr. Top. Microbiol. Immunol.* 1984. *113:* 231.
14 Ford, C. E., Hamerton, J. L., Barnes, D. W. and Loutit, J. F., *Nature* 1956. *177:* 452.
15 Miller, J. F. A. P. and Mitchell, G. F., *Transplant. Rev.* 1969. *1:* 3.
16 Abramson, S., Miller, R. G., and Phillips, R. A., *J. Exp. Med.* 1977. *145:* 1567.
17 Toivanen, P. and Toivanen, A., *Eur. J. Immunol.* 1973. *3:* 585.
18 Osmond, D. G. and Nossal, G. J. V., *Cell. Immunol.* 1974. *13:* 132.
19 Nossal, G. J. V. and Pike, B. L., *Immunology* 1973.
20 Strober, S., *Transplant. Rev.* 1975. *24:* 84.
21 Landreth, K. S., Rosse, C. and Clagett, J., *J. Immunol.* 1981. *127:* 2027.
22 Pink, R. J., Ratcliffe, M. J. H. and Vainio, O., *Eur. J. Immunol.* 1985. *15:* 617.
23 Ratcliffe, M. J. H., *Immunol. Today* 1985. *6:* 223.