Kevin Flurkey●, Miguel Stadecker▲ and Richard A. Miller

Department of Pathology, Institute of Gerontology● and VA Medical Center, University of Michigan, Ann Arbor and Department of Pathology▲, Tufts University School of Medicine, Boston

Memory T lymphocyte hyporesponsiveness to non-cognate stimuli: a key factor in age-related immunodeficiency*

Previous studies from our laboratory have suggested that aging leads to an accumulation of cells expressing high levels of CD44, thought to be a marker for memory lymphocytes, and that positively selected $CD44^{hi}$ T cells, from mice of any age, respond poorly to concanavalin A (Con A) in limiting dilution estimates of interleukin (IL)-2-producing cells. We now report the results of a more comprehensive analysis of memory T cell function, in old and young mice, to non-cognate activators (Con A and the staphylococcal enterotoxin SEB). We report that memory T cells, isolated by removing cells bearing the CD45RB determinant, contain very few cells able to respond to either Con A or SEB under limiting dilution culture conditions, whether the responses are measured by IL-2 or by IL-3 accumulation. As a control, we show that memory T cells do respond strongly, at limiting dilution, to recently encountered priming antigens, *i.e.* *Schistosoma mansoni* egg antigen; the limiting dilution culture protocol thus does not preclude activation of memory T cells when cognate stimuli are presented to antigen-specific cells. These data suggest that virgin and memory T cells may differ fundamentally in their activation requirements, and suggest further that the accumulation, with age, of memory T cells accounts for the low responsiveness of old mice to non-cognate mitogens.

1 Introduction

The decline, with age, in the T cell proliferative response to plant lectins is the most robust and thoroughly analyzed observation in immunogerontology [1]. This decline seems likely to reflect both a diminished production of IL-2 [2] and a decrease in the number of T cells that can respond to mitogens by production of functional, high-affinity IL-2 receptors [3, 4]. LD assays have suggested that aging leads to a decline in the proportion of T cells that can generate IL-2 in response to Con A, without detectable alteration in the amount of IL-2 produced by each reactive cell [4]. An age-related shift to a predominance of memory T cells [5–7] may play a key role in the loss of Con A-responsive cells. Positively selected $CD44^{hi}$ (memory) cells from young or old mice respond poorly in LD tests for IL-2 production, proliferation and generation of cytotoxic effectors [5] compared to $CD44^{low}$ cells. Furthermore, positively selected $CD45RB^{hi}$ (naive) T cells, from young or old mice, produce more IL-2 than $CD45RB^{low}$ cells in conventional high density cultures stimulated with Con A [7]. However, interpretation of results from positively selected cell populations is complicated by the possibility that the antibodies used for selection may influence responses. Therefore, in the current experiments, our first goal was to compare directly the frequency of Con A-responsive, IL-2- and IL-3-producing T cells in negatively selected naive and memory T cells from young and old mice. Second, to see if the age-related decline in frequency of responsive T cells can be detected when triggering is T cell receptor mediated, we tested naive and memory T cells in a newly devised LD test for responses to staphylococcal enterotoxin B (SEB).

[I 9778]

* This research was supported by NIA grant AG03978 and by a grant to Kevin Flurkey from the American Federation for Aging Research.

Correspondence: Richard A. Miller, University of Michigan, Box 2007, 300 N. Ingalls Street, Ann Arbor, MI 48109, USA

Abbreviation: **SMEA:** *Schistosoma mansoni* egg antigen

2 Materials and methods

2.1 Mice and reagents

Young and middle-aged (C57BL/6 × CBA)F_1 (B6CBAF_1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), or bred in our laboratory from Jackson Laboratory stock. C3HeB/FeJ female mice (6–8 weeks old) were obtained from The Jackson Laboratory and infected with 60 cercariae of *S. mansoni* (Puerto Rico strain) at the Center for Tropical Diseases, Lowell, MA. They were maintained in a colony at Tufts University, Boston, until being killed at 3–4 months of age, 8 weeks after infection. Reagents and monoclonal antibodies used were described in a previous publication [8].

2.2 Cell preparation and sorting

The methods used have been described previously [8], except that in the current work cells were stained at 4×10^6/ml, and anti-CD44 antibody was used at 1:100. Cells were classified as $CD44^{low}$ or $CD45RB^{low}$ if they were within the lowest quartile for the respective marker. "Mock-sorted" cells were collected from the same preparations without regard to a fluorescence sorting criterion. Forward and 90° scatter gates were used to exclude

0014-2980/92/0404-0931$3.50+.25/0

non-lymphoid cells. The proportion of cells that were CD4$^+$ was determined in all sorted and control samples.

2.3 LD analysis

The methods for estimating the frequency of Con A-inducible precursors for IL-2-secreting helper T cells ("pIL-2 assay") and IL3-secreting helper cells ("pIL-3 assay") have been described previously [5]. LD assays for SEB- and *S. mansoni* egg antigen (SMEA)-specific helper T lymphocyte precursor (pHTL) were set up exactly as for Con A pHTL, except that SEB (1 µg/ml) or SMEA (5 µg/ml) was substituted for Con A, and the cultures were incubated for 5 days before supernatants were harvested for IL-2 and IL-3 determinations.

3 Results

3.1 Expression of CD44 and CD45RB on T cells from old and young mice

The proportion of B6CBAF$_1$ CD4$^+$ splenocytes that were CD44^{hi} increased from 27 ± 3 at 2–6 months of age to 49 ± 6 at 19–24 months (mean ± SEM; $n = 4$ pairs; $p < 0.05$), while the proportion that were CD45RB^{hi} decreased from 72 ± 4 to 36 ± 4 ($n = 5$ pairs; $p < 0.001$). These changes are similar to those reported for other strains of mice [5–7]. Most cells expressed either high levels of CD45RB or high levels of CD44; typically, <10 % of the cells expressed high levels of both markers and <20 % of the cells expressed only low levels of both markers in both young and old mice (not shown).

3.2 LD analyses of IL-3-producing cells and of responses to the superantigen SEB

Our previous work has shown an age-related decline in the proportion of murine splenic T cells that could generate IL-2 in LD cultures when activated with Con A. In the current studies we sought to determine if there was a similar age-related decline in the proportion of T cells that could generate a second lymphokine (IL-3), and in the proportion of cells that respond to SEB, an antigen known to trigger T cells via their antigen receptor. Fig. 1 illustrates the dose-response curve for proliferation of mouse splenocytes induced by SEB at high culture densities and shows that the level of proliferation is reduced by aging. In LD cultures (Table 1) young mice had 2.5- to 3.7-fold higher proportions of responsive T cells than did old mice; the differences are statistically convincing for production of both IL-2 and IL-3 in responses to Con A, and for SEB-induced IL-2 production. There was a clear age-dependent trend (3.5-fold) towards lower frequencies of SEB-reactive IL-3-producing cells as well, with higher responses from the young mice in four of the five paired experiments, but the difference observed was not statistically significant. Since the proportion of T cells that express V$_\beta$8 and the density of V$_\beta$8 on the cell surface does not change with age in mice ([9] and R. Eren and R. Miller, unpublished), age-associated reductions in SEB-activated pHTL probably result from accumulations of unresponsive V$_\beta$8$^+$ T cells.

3.3 Comparisons of naive and memory T cells by LD analysis

We have previously observed [5] a relatively high frequency of Con A-responsive pIL-2 cells among naive T cells, selected for low expression of CD44 (Pgp-1), compared to memory T cells selected for high CD44 expression for both young and old mice [5]. We now report that this distinction between naive and memory T cells extends to IL-3 secretion as well, and to responses triggered by the SEB/TcR interaction. Selection against high expression of CD45RB cells produced CD45RB^{low} populations consisting largely (*i.e.* >90 %) of CD4$^+$, CD44^{hi} cells, since most murine leukocytes, including CD8$^+$ cells, express the B exon of the CD45R antigen [10], and so are removed by our selection procedure. CD45RB^{low} preparations from young mice typically had somewhat higher levels of contamination by CD44^{low} cells than preparations from old mice.

Table 1. Effect of age on helper T cell frequencies: young/old ratios for responses to Con A and SEB[a)]

Stimulus	n	pIL-2	pIL-3
Con A	7	2.94 ± 0.70[b)]	3.74 ± 0.79[b)]
SEB	5	2.48 ± 0.56[c)]	3.52 ± 1.05

a) Values given are means ± SEM of young-to-old ratios obtained for splenocyte samples from n individual paired LD experiments. Three of the Con A experiments and three of the SEB experiments represent the unsorted control samples also shown in Figs. 2 and 3. The remaining experiments produced mean pHTL frequencies (expressed per 10^3 Thy-1 cells) for young mice as follows: Con A: 311 pIL-2, 122 pIL-3; SEB: 62 pIL-2, 44 pIL-3.
b) $p < 0.01$ for hypothesis that young = old by paired t-test
c) $p < 0.05$ for hypothesis that young = old by paired t-test

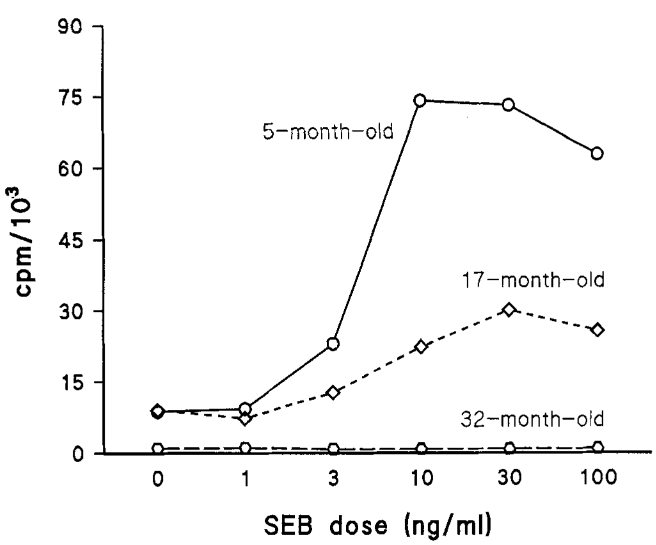

Figure 1. SEB-induced proliferation of splenocytes from mice of different ages. Data shown are means of triplicate wells of 2 × 10^5 spleen cells cultured in flat-bottom wells with SEB. Cells were harvested on the fifth day of culture 6 h after 0.5 µCi [^{3}H]thymidine was added per well.

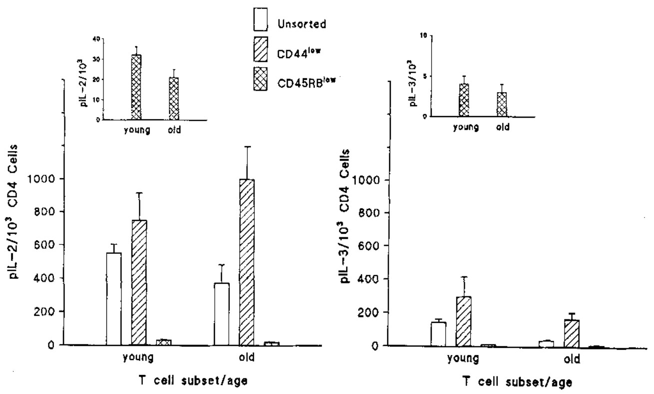

Figure 2. Preferential activation of naive T cells by Con A. pIL-2 (left panel) and pIL-3 values (right panel) represent means (+ SEM) of three experiments, each involving splenocyte samples from one young (3-to-6-month) and one old (19-to-20-month) female mouse, and are expressed per 10^3 $CD4^+$ cells for reasons discussed in the text. The frequencies of pIL-2 and pIL-3 were higher among $CD44^{low}$ cells ($p < 0.01$ for pIL-2 cells and $p < 0.05$ for pIL-3) and lower among $CD45RB^{low}$ cells ($p < 0.001$) compared to unsorted controls (ANOVA followed by planned comparisons of cell subsets, age groups combined, using log-transformed data). Data for unsorted cells from young and old mice are provided for comparison to other cell subsets; see Table 1 for the evaluation of age effects. Insets show precursor frequencies of the $CD45RB^{low}$ cells on an expanded scale.

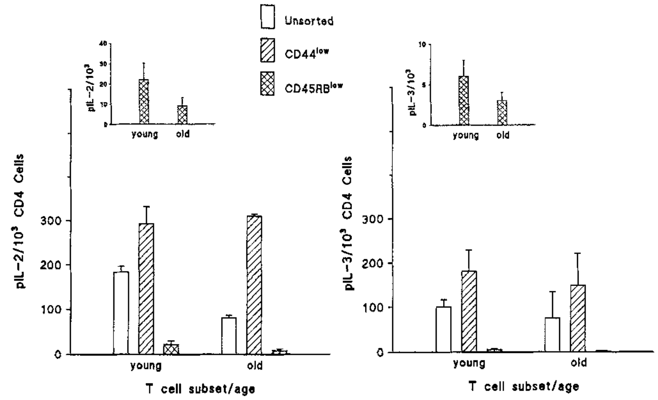

Figure 3. Preferential activation of naive T cells by SEB. pIL-2 (left panel) and pIL-3 values (right panel) represent means (+ SEM) of three experiments, each involving splenocyte samples from one young (3-to-6-month) and one old (19-to-20-month) female mouse, expressed per 10^3 $CD4^+$ cells. The frequencies of pIL-2 and pIL-3 were enhanced in the $CD44^{low}$ subset ($p < 0.05$) and diminished in the $CD45RB^{low}$ subset ($p < 0.001$) compared to controls (ANOVA, planned comparisons; log-transformed data). See Table 1 for evaluation of age effects.

Both Con A- and SEB-responsive cells were found to be severely depleted in the memory cell subset, and enriched in the naive cell subset, whether IL-2 or IL-3 was used as the index of helper cell function (Figs. 2 and 3; $p < 0.001$). In contrast, the proportion of naive ($CD44^{low}$) T cells that can generate IL-2 in short-term LD cultures was essentially the same as the fraction of CD4 cells in both young and old mice, suggesting that nearly every $CD4^+$ T cell was responsive to Con A under these culture conditions. Additionally, the proportion of SEB-responsive cells within the naive pool (25 %–30 % of the $CD4^+$ cells) was consistent [9, 11] with the fraction of T cells that express the complementary $V_{\beta}8$ and $V_{\beta}3$ chains. Results are presented as the frequency of responders per $CD4^+$ cell since we have previously shown that only $CD4^+$ T cells generate IL-2 in LD cultures [12], and since negative selection using anti-CD45RB removes most or all $CD8^+$ cells.

These results thus confirm, using negative selection methods, our earlier report that there are differences between naive and memory T cell responsiveness under LD conditions but little or no decline in the proportion of naive cells that can respond to Con A by making IL-2 in LD cultures [5].

3.4 Responses of primed mice to SMEA

The results above suggested that few memory T cells can respond to Con A or SEB by production of IL-2 or IL-3. We needed, however, to consider the possibility that the LD conditions used were unsuitable for activation of memory T cells under any circumstances. We, therefore, tested the responses, in LD cultures, of T cells from young mice to an antigen to which the donors had been recently primed. We have shown previously that splenic and lymph node cells from mice infected with *S. mansoni* will secrete IL-2 in LD cultures when challenged with SMEA [8]. Table 2 presents the results of a more extensive series of experiments, utilizing IL-3 assays to minimize possible effects of lymphokine utilization in culture, in which lymph node cells from *S. mansoni*-infected young mice were sorted into naive ($CD44^{low}$) and memory ($CD45RB^{low}$) subsets and then challenged in LD cultures with SMEA. The Con A results showed that the priming procedure did not alter the high

Table 2. Con A- and SMEA-Responsive pIL-3 in subsets of T cells from schistosome-infected mice[a)]

Stimulus	Unsorted controls	Anti-CD44 mock sort	Anti-CD45RB mock sort	$CD44^{low}$	$CD45RB^{low}$
SMEA	6.2 ± 1.8	6.0 ± 2.0	6.7 ± 2.5	1.7 ± 0.5[b)]	12.3 ± 4.7
Con A	189 ± 62	152 ± 58	93 ± 29	434 ± 135	16 ± 6[c)]

a) Values are mean (± SEM; $n = 3$) frequencies of pIL-3 per 10^3 $CD4^+$ mesenteric lymph node cells from mice infected with *S. mansoni* 8 weeks before being killed. Mock-sorted control preparations were made by treatment with the indicated antibody and passed through the cell sorter but without reference to the fluorescent signal. Unsorted control cells were neither exposed to antibody nor processed through the cell sorter. Sorted (and control) cell preparations were also tested in the absence of stimulus to measure spontaneous IL-3 production; frequencies estimated from these unstimulated controls ranged from 1.0×10^3–1.7×10^3 $CD4^+$ cells. Unsorted cells from uninfected control mice yielded Con A pIL-3 frequencies of 171 ± 40 and SMEA-reactive frequencies of 1.1 ± 0.5.

b) Significantly less than the values for unsorted controls and $CD45RB^{low}$ cells ($p < 0.05$).

c) Significantly less than the values for unsorted controls and $CD44^{low}$ cells; ($p < 0.05$).

ratio of naive to memory Con A-responsive pIL-3 cells. The SMEA-stimulated cultures, however, showed just the reverse pattern: SMEA-responsive pIL-3 were about 7-fold more frequent in the memory cell subset than in the naive subset. Mock-sorted controls, included in each experiment, showed that the differences between the naive and memory cell populations cannot be ascribed simply to an artifact of exposure to the antibodies or sorting machinery.

We conclude that antigen-specific memory lymphocytes, enriched on the basis of differential CD44/CD45RB expression, do indeed respond well, compared to virgin T cells, to cognate antigen under LD culture conditions. The differences in responses to non-cognate stimuli, *i.e.* to Con A and SEB, cannot simply be explained as an inability of the LD cultures to support activation of memory T cells.

4 Discussion

Our results provide new insights both into the immunodeficiency of aging and into functional differences between naive and memory T cells. Our demonstration of an age-related decline in the frequency of T cells that can respond to SEB by IL-2 production clarifies ambiguities left unresolved by previous work [5, 7] on Con A responses. Con A reacts with many surface glycoproteins, and it is unclear whether its effect on the TcR is the only interaction mediating T cell activation by this mitogen. The new data using SEB as a stimulus thus extend the LD findings to an activator known to stimulate via the TcR.

The 3.7-fold decline, with age, in Con A-responsive pIL-3 shows that aging leads to diminished secretion of this lymphokine in addition to IL-2. The published data on IL-3 production by aging T cells are conflicting and inconclusive. Two groups [13, 14] have used conventional high-density culture methods to show a decline in Con A-induced IL-3 secretion by older mice. Kubo and Cinader, on the other hand, have presented results of an LD study [15] using PMA and calcium ionophore as activating agents, in which they report an increase in pIL-3 cell number in older mice. It is worth noting, however, that the frequencies of pIL-3 measured by this group (0.05–$0.17/10^3$ T cells) were about 1000-fold lower than those detected with our own Con A method, and probably provide little or no information about the functional capabilities of the cells. Results from Kubo and Cinader [15] and Iwashima et al., [16], using Con A in high-density cultures, indicate that splenocytes from old mice produce more IL-3 within the first 24 h of activation, although this is several days before the time of maximal IL-3 accumulation observed in our own laboratory. On balance, we suspect that aging probably does lead to a decline in IL-3 accumulation under standard test conditions, but additional analysis is still needed.

The central focus of the present work was the comparison of memory and virgin T cell responses to Con A and SEB. The data (Figs. 2 and 3) clearly show that virgin T cells respond well, and memory T cells respond very poorly, to each of these non-cognate stimuli under LD conditions, regardless of the lymphokine measured as an index of helper T cell activation. The results are consistent with, and suggest an explanation for, the observation that proliferation of naive human peripheral blood T cells in response to plant lectin mitogens is usually higher than proliferation of memory T cells [17–20]. The SMEA results (Table 2) demonstrate, as in important control, that the LD culture conditions can, for appropriate stimuli (*e.g.* cognate antigens presented to recently primed responders), support lymphokine secretion by memory T cells. It is unlikely that differential absorption contributes to the differences we observe between virgin and memory cells, since expansion of IL-2-dependent clones is minimal under our conditions, and since the IL-2 results are closely paralleled by the IL-3 data.

Memory T cells have been reported to respond better than naive T cells, by proliferation [21–23] and IL-3 production [24, 25], to a variety of non-lectin stimuli, or to combinations of lectins plus phorbol esters. This has led to the suggestion that memory cells may be easier to activate than naive cells, and that IL-3 in particular may be preferentially produced by memory cells [21, 26]. All the available data from these studies are from high-density cultures, which may be necessary in some cases for production of detectable levels of lymphokine. Interpretation of such data is difficult since, unlike LD tests, it is not clear that the overall response is determined primarily by the effector cell being tested. Our LD data ([5] and this report), together with our previous demonstration that $CD4^+$ memory T cells are more resistant than virgin T cells to agents that induce intracellular calcium signals [27], and our present observation that IL-3 is not produced exclusively by memory cells, suggest an alternative hypothesis. We propose that memory T cells may indeed be easier to trigger by stimuli that mimic representation of the original, priming cognate antigen – *e.g.* anti-CD3 plus anti-CD4, or the antigen itself – but may be less responsive to agents that provide only a poor imitation of the priming antigen, including plant lectins and SEB.

The status of SEB in this scheme is somewhat unclear. Horgan et al. [26] have shown that naive T cells from human peripheral blood proliferate poorly in response to SEB and SEA at doses (0.1–1000 ng/ml) lower than the dose (1000 ng/ml) we found to be optimal for our LD cultures. In contrast, Dohlsten et al. [28] reported stronger proliferation of human naive cells than of memory cells, in bulk cultures, to the closely related SEA molecule, despite a more rapid production of IL-2 by the latter cell subset. A disparity between the results of bulk culture assays and LD tests for frequency, if indeed one does apply to SEB responses of murine splenocytes, would not be unprecedented [29, 30].

Our work leaves unanswered the question of whether memory T cells, in old mice, are as frequently responsive as memory T cells taken from younger animals, since the agents we used for activation were relatively ineffective with memory cells. Our current data, like that in [5], show a small decline with age in pHTL frequencies within the memory cell population in responses triggered by non-cognate stimuli, although the 2%–10% contamination of the $CD45RB^{low}$ preparations by $CD44^{low}$ T cells could account for much or all of the observed response. Whatever the effect of age on memory cell pHTL frequencies, it can account for no more than about 10% of the overall age-effect on Con A-responsive pHTL numbers since memory cell pHTL frequencies are so low, even in young mice. The poor response of old mice to stimuli like Con A and SEB seems to reflect an accumulation of a cell type – the memory T cell – which is hyporesponsive to these agents in mice of any age.

We thank Drs. Bottomly, Hyman, Marshak-Rothstein, and Raulet for their kind donations of antibody or hydridoma cell lines, and Gary Weil for assistance in the cell sorting experiments.

Received July 17, 1991; in revised form December 2, 1991.

5 References

1 Miller, R. A., *Int. Rev. Cytol.* 1991. *124:* 187.
2 Thoman, M. L. and Weigle, W. O., *Adv. Immunol.* 1989. *46:* 221.
3 Hara, H., Tanaka, T., Negoro, S., Deguchi, Y., Nishio, S., Saiki, O. and Kishimoto, S., *Mech. Ageing Dev.* 1988. *45:* 167.
4 Miller, R. A., *J. Immunol.* 1984. *132:* 63.
5 Lerner, A., Yamada, T. and Miller, R. A., *Eur. J. Immunol.* 1989. *19:* 977.
6 Ernst, D. N., Hobbs, M. V., Torbett, B. E., Glasebrook, A. L., Rehse, M. A., Bottomly, K., Hayakawa, K., Hardy, R. R. and Weigle, W. O., *J. Immunol.* 1990. *145:* 1295.
7 Nagelkerken, L., Hertogh-Juijbregts, A., Dobber, R. and Drager, A., *Eur. J. Immunol.* 1991. *21:* 273.
8 Miller, R. A., Flurkey, K., Molloy, M., Luby, T. and Stadekker, M. J., *J. Immunol.* 1991. *147:* 3080.
9 White, J., Herman, A., Pullen, A. M., Kubo, R., Kappler, J. W. and Marrack, P., *Cell* 1989. *56:* 27.
10 Bottomly, K., Luqman, M., Greenbaum, L., Carding, S., West, J., Pasqualini, T. and Murphy, D. B., *Eur. J. Immunol.* 1989. *19:* 617.
11 Ernst, D. N., Weigle, W. O., McQuitty, D. N., Rothermal, A. L. and Hobbs, M. V., *J. Immunol.* 1989. *142:* 1413.
12 Miller, R. A., *J. Immunol.* 1983. *131:* 2864.
13 Li, D. D., Chien, Y. K., Gu, M. Z., Richardson, A. and Cheung, H. T., *Life Sci.* 1988. *43:* 1215.
14 Chang, M. P., Utsuyama, M., Hirokawa, K. and Makinodan., T., *Cell. Immunol.* 1988. *115:* 1.
15 Kubo, M. and Cinader, B., *Eur. J. Immunol.* 1990. *20:* 1289.
16 Iwashima, M., Nakayama, T., Kubo, M., Asano, Y. and Tada, T., *Int. Arch. Allergy Appl. Immunol.* 1987. *83:* 129.
17 Sanders, M. E., Makgoba, M. W., Sharrow, S. O., Stephany, D., Springer, T. A., Young, H. A. and Shaw, S., *J. Immunol.* 1988. *140:* 1401.
18 Morimoto, C., Letvin, N. L., Boyd, A. W., Hagan, M., Brown, H. M., Kornacki, M. M. and Schlossman, S. F., *J. Immunol.* 1985. *134:* 3762.
19 Morimoto, C., Letvin, N. L., Distaso, J. A., Aldrich, W. R. and Schlossman, S. F., *J. Immunol.* 1985. *134:* 1508.
20 Tedder, T. F., Cooper, M. D. and Clement, L. T., *J. Immunol.* 1985. *134:* 2989.
21 Sanders, M. E., Makgoba, M. W. and Shaw, S., *Immunol. Today* 1988. *9:* 195.
22 Byrne, J., Butler, J. L., Reinherz, E. L. and Cooper, M. D., *Int. Immunol.* 1989. *1:* 29.
23 Anderson, P., Blue, M. L., Morimoto, C. and Schlossman, S. F., *J. Immunol.* 1987. *139:* 678.
24 Budd, R. C., Cerottini, J.-C. and MacDonald, H. R., *J. Immunol.* 1987. *138:* 3583.
25 Swain, S. L., Weinberg, A. D. and English, M., *J. Immunol.* 1990. *144:* 1788.
26 Horgan, K. J., Van Seventer, G. A., Shimizu, Y. and Shaw, S., *Eur. J. Immunol.* 1990. *20:* 1111.
27 Philosophe, B. and Miller, R. A., *J. Gerontol. Biol. Sci.* 1990. *47:* B87.
28 Dohlsten, M., Hedlund, G., Sjögren, H.-O. and Carlsson, R., *Eur. J. Immunol.* 1988. *18:* 1173.
29 Rozans, M. K., Smith, B. R., Burakoff, S. J. and Miller, R. A., *J. Immunol.* 1986. *136:* 4040.
30 Miller, R. A. and Reiss, C. S., *J. Mol. Cell. Immunol.* 1984. *1:* 357.