INTERLEUKIN 2 RECEPTOR EXPRESSION IN UNSTIMULATED MURINE SPLENIC T CELLS

Localization to L3T4$^+$ Cells and Regulation by Non-H-2-Linked Genes

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T cell growth factor, now commonly referred to as IL-2 (1, 2), interacts with the specific receptors on the cell surface to mediate its function. The existence of IL-2-R has been shown by the absorption or the specific binding of IL-2 by activated T cells; however, these have not been shown for resting T cells (3–5). Recently, mAbs that detect IL-2-R were made by several laboratories (6–8). Generally, it has been believed to be difficult to detect a significant population of IL-2-R-bearing T cells in unstimulated spleen cells from unimmunized mice (3, 5, and 6). After nonspecific stimulation in vitro, IL-2-R-bearing T cells are detected in both the L3T4$^+$Lyt-2$^-$ and L3T4$^-$Lyt-2$^+$ subpopulations in the mouse (9). A small population of IL-2-R-bearing cells has been found among unstimulated thymocytes, but these appear to be exclusively immature L3T4$^-$Lyt-2$^-$ thymocytes (10, 11).

In the course of studying the effect of nonimmune T cells contaminating irradiated spleen cells used as APCs, we discovered a non-H-2-linked strain difference in the ability of unstimulated splenic T cells to respond to high doses of IL-2. This observation led us to search for possible strain differences in IL-2-R expression in unstimulated splenic T cell populations. In the course of these studies, reported here, we made several novel observations. First, flow cytometry (FCM)[1] analysis of highly enriched preparations of T cells from unstimulated, unimmunized fresh spleen cells revealed a distinct positive population of 3–7% consisting of cells bearing IL-2-R. Second, contrary to expectations from studies of stimulated T cells, these cells were confined to the L3T4$^+$Lyt-2$^-$ subset. No IL-2-R$^+$ cells were found in the Lyt-2$^+$ population, in contrast to observations after stimulation in vitro (reference 9 and current results). Third, a twofold difference in the relative proportion of the receptor-positive population was reproducibly observed among strains, quantitatively consistent with the magnitude of difference in responsiveness to IL-2. Although multiple non-H-2-linked genes appear to play a role in this strain difference, the data from recombinant inbred (RI) lines of mice suggest that at least one gene of major impact may map to chromosome 7.

[1] *Abbreviations used in this paper:* FCM, flow cytometry; IF, immunofluorescence; MAR 18.5, mouse anti–rat kappa chain mAb; RI, recombinant inbred; TRA, Texas red–conjugated avidin.

Materials and Methods

Animals. B10.D2/nSn, B6D2F$_1$, BALB/cJ, DBA/2J, C57BL/6 (B6), and 23 B × D RI strains were purchased from The Jackson Laboratory (Bar Harbor, ME) and were 8–20 wk old at the time of testing.

Reagents. The anti–IL-2-R antibody 7D4 has been described previously (6, 9, 12, and 13). 7D4 culture supernatant and FITC-conjugated 7D4 were gifts of Drs. T. Malek and E. Shevach, and Dr. G. Shearer (NIH), respectively. FITC-mouse anti–rat kappa chain mAb (MAR 18.5), FITC-anti-Thy-1.2 (30-H12), and biotin-conjugated anti-Lyt-2 (53-6) were purchased from Becton-Dickinson Immunocytometry Systems (Mountain View, CA). Biotin-conjugated monoclonal anti-L3T4 (H129.19) (14) and Texas red–conjugated avidin (TRA) (15) were gifts of J. Titus (NIH). Biotin and fluorescein-conjugated OKT8 were gifts of Dr. Ralph Quinones (NIH). The rIL-2 was kindly supplied by the Cetus Corp. (Emeryville, CA), and has been characterized previously (16). EL-4 culture supernatant was prepared by stimulating EL-4 T lymphoma cells at 10^6/ml (a gift of Drs. M. Doyle and D. F. Mark, Cetus Corp.), with 50 ng/ml mezerein (a gift of Dr. Irwin Braude, Meloy Laboratories Inc., Springfield, VA), for 48 h; centrifugation to remove cells; and dialysis to remove mezerein.

Preparation of T Cells. T cells were prepared by passing through a nylon wool column as described (17). For maximal purity, the following conditions were used: 1 g of nylon wool was employed per spleen, preequilibrated with RPMI 1640 with 10% FCS, and prewarmed to 37°C. The cells were incubated on the column for 45 min at 37°C and eluted with 6–7 ml of warmed (37°C) medium. Where indicated, Con A stimulation was performed by culturing 10^6/ml unseparated spleen cells with 14.5 μg/ml Con A for 48 h in culture flasks, according to the method of G. Shearer (personal communication).

Proliferation Assay. For IL-2 responses, 5×10^4 T cells or spleen cells were cultured in complete medium (18) in the presence of various concentrations of IL-2 for 2 d (except as indicated in the figures) at 37°C, with 5% CO_2 in 96-well plates (0.2 ml/well) before determination of [^{3}H]thymidine incorporation. During the last 24 h, 1 μCi [methyl-^{3}H]-thymidine (6.7 Ci/mol; New England Nuclear, Boston, MA) was added. Cells were harvested on an automated device (Skatron, Inc., Sterling, VA), and proliferation was estimated by scintillation counting of the ^{3}H-cpm incorporated into DNA.

Immunofluorescence (IF) Staining and FCM Analysis. For indirect one-color staining, 10^6 T cells prepared as described above were treated with 7D4 culture supernatant for 40 min at 4°C, washed twice, treated with FITC-MAR 18.5 for 40 min, washed twice, and resuspended for FCM analysis. Alternatively, 10^6 T cells were incubated with FITC-anti-Thy-1.2, washed twice, and resuspended for FCM analysis. For two-color IF staining, 10^6 T cells were incubated at 4°C for 40 min with FITC-7D4, washed twice, incubated with biotin-labeled anti-L3T4 or anti-Lyt-2, washed twice, and subsequently incubated with TRA for 10 min, followed by washing and resuspension. These procedures were performed using HBSS without phenol red, containing 0.1% BSA and 0.1% sodium azide. All reagents were used at concentrations previously determined to be saturating.

FCM analysis was performed as described previously (19) using a B-D Dual Laser (argon, dye; Becton-Dickinson & Co., Sunnyvale, CA) FACS II. FITC was excited at 488 nm and TRA was excited at 590 nm. Data on individual cells were collected, stored, displayed, and analyzed using a PDP 11/24 computer (Digital Equipment Corporation, Marlboro, MA) interfaced to the FACS II (20). Fluorescence data were collected using logarithmic amplification on 50,000 viable cells, as determined by forward light scatter intensity. Logarithmic amplification was provided by a 3-decade logarithmic amplifier constructed from an NIH-modified design of R. Hiebert, Los Alamos Scientific Laboratories (Los Alamos, NM). One-color fluorescence data were displayed as cell frequency histograms or IF profiles in which log fluorescence intensity was plotted in 1,024 channels on the *x*-axis and cell number shown on the *y*-axis. Two-color IF data were displayed as contour diagrams in which log intensities of green (FITC) fluorescence were plotted in 64 channels on the *x*-axis and log intensities of red (TRA) fluorescence were plotted on the *y*-axis. Levels parallel to the *x*,*y* plane were selected on the *z*-axis (the number of cells

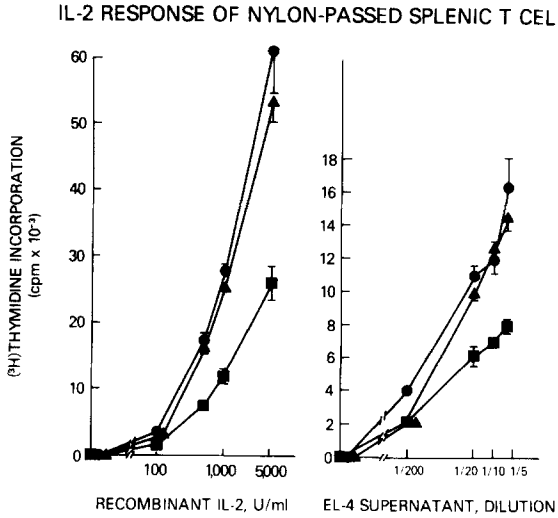

FIGURE 1. 5×10^4 nylon wool column–passed spleen cells from BALB/c (▲), DBA/2 (●), or B10.D2 (■) were cultured with various concentrations of rIL-2 or EL-4 culture supernatant containing IL-2 for 48 h. The EL-4 supernatant contained about 680 U/ml of IL-2 by titration on IL-2-dependent HT2A cells in comparison with rIL-2 standard. Means and standard errors of triplicates are indicated.

at any intersection of x and y values). These slices through peaks resulted in contours correlating red and green fluorescence.

All data were analyzed for minimum numbers of possible cells by integration of IF profiles above a selected fluorescence intensity. For each experiment this level of fluorescence intensity was selected as the intersection of the IF profile obtained from DBA/2 T cells stained with 7D4 and FITC-MAR 18.5, and the profile from the same cells stained with FITC-MAR 18.5 only. This cutoff level was used for all data in that experiment. The percent positive cells reported in this study equals [percent positive (7D4 + FITC-MAR 18.5) − percent positive (FITC-MAR 18.5 only)]. It should be noted that this analysis determines only the minimum number of cells that can be detected by IF, and there may be positive cells with fluorescence below the detection limits of the instrument.

Results

We examined the ability of rIL-2 or IL-2-containing EL-4 supernatant to stimulate proliferation of nylon-passed splenic T cells freshly removed from unimmunized mice (Fig. 1). As expected from previously published experiences (1, 5–9, and 21–24), no proliferation was induced in splenic T cells from unimmunized mice by conventional doses of IL-2. However, very high concentrations of rIL-2 (or EL-4 culture supernatant) induced significantly more proliferation by DBA/2 or BALB/c T cells than by B10.D2 T cells (Fig. 1). This observation was quite reproducible in other experiments (not shown). To determine whether this difference in responsiveness to IL-2 was due to a difference in the number of IL-2-sensitive cells in unstimulated splenic T cell populations, we titrated the number of responding nylon-passed spleen T cells at a constant dose of rIL-2 (500 U/ml, based on results shown in Fig. 1). The response curve for B10.D2 spleen cells was shifted to the right relative to those of DBA/2 and BALB/c (Fig. 2). It required approximately twice as many B10.D2 T cells as

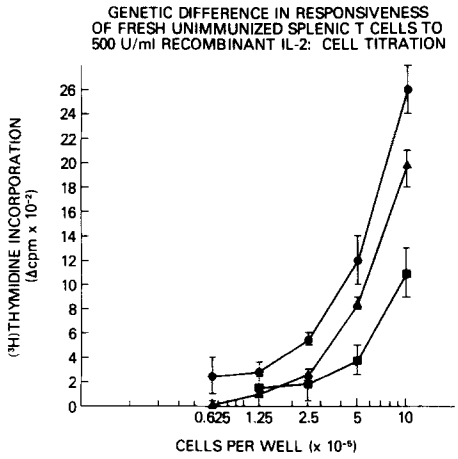

FIGURE 2. Cell titration showing a twofold difference in the number of unstimulated T cells necessary to obtain a given response to high-dose IL-2. Fresh, unstimulated spleen cells from unimmunized mice of strains DBA/2 (●), BALB/c (▲), and B10.D2 (■) were passed over a nylon wool column, as described in Materials and Methods, to purify T cells, and then were cultured at the indicated cell numbers in microtiter wells with or without 500 U/ml of Cetus rIL-2 for 48 h at 37° in 5% CO_2. 1 μCi of [^{3}H]thymidine was added to each well 6 h before harvest. The ordinate shows the difference in [^{3}H]thymidine incorporated between means of pairs of triplicate cultures with and without IL-2 for each cell number and strain. The backgrounds without IL-2 were generally 100–300 cpm depending on cell number, and did not vary with strain. Error bars are SEM.

DBA/2 T cells to produce the same level of response. Thus, the difference in responsiveness appeared to be due to a difference in the number of cells responding. Therefore, we asked whether there was a strain difference in the fraction of T cells bearing receptors for IL-2 before stimulation.

The Presence of IL-2-R$^+$ T Cells in Uncultured Spleen Cells from Unimmunized Mice and Strain Differences in the Fraction of Positive Cells. Since previous studies (6, 9) had not reported a significant number of IL-2-R-bearing cells in unstimulated spleen cells from unimmunized mice, we used highly enriched T cell populations to enhance the detection of low frequency cells by FCM. Passage of fresh spleen cells over large nylon wool columns resulted in populations that were ~90–95% Thy-1.2$^+$ by IF staining (Fig. 3, *b* and *d*). Staining of these cells with 7D4 monoclonal anti-IL-2-R antibody, followed by FITC-MAR 18.5 monoclonal anti-rat kappa chain, revealed a small but distinct shoulder of positively staining cells compared with the cells stained with MAR 18.5 alone (Fig. 3, *a* and *c*). Moreover, the shoulder was distinctly larger in DBA/2 T cells (*a*) than in B10.D2 T cells (*c*). This difference was quite reproducible (Fig. 4). Five mice from each strain were tested individually and showed minimal variation among mice of the same strain but consistent differences between strains (Fig. 4*A*). The mean percent IL-2-R$^+$ cells was 6.25 ± 0.24 SEM for DBA/2, 6.04 ± 0.18 for BALB/c, and 3.05 ± 0.23 for B10.D2 ($p < 0.001$ for B10.D2 vs. DBA/2; $p < 0.001$ for B10.D2 vs. BALB/c; and DBA/2 vs. BALB/c, NS). Even when five other experiments on different days were compiled, in which only one mouse of each strain was tested per experiment (as controls for the RI lines, see below), the groups were almost as tight and the strain differences quite reproducible

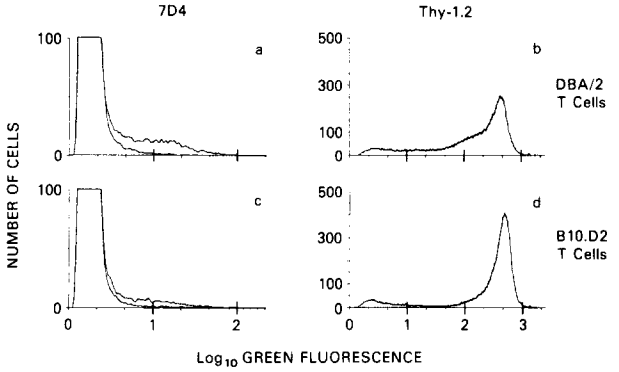

FIGURE 3. IF profiles of DBA/2 T cells (*a* and *b*) and of B10.D2 T cells (*c* and *d*). In experiments shown in *a* and *c* cells were treated with 7D4 plus FITC-MAR 18.5 (upper curves of each panel) or with FITC-MAR 18.5 alone (lower curves of each panel), whereas in *b* and *d* cells were treated with FITC-Thy-1.2. In *a* and *c* the ordinate (cell number) and abscissa (fluorescence intensity) have been expanded to show the region of interest.

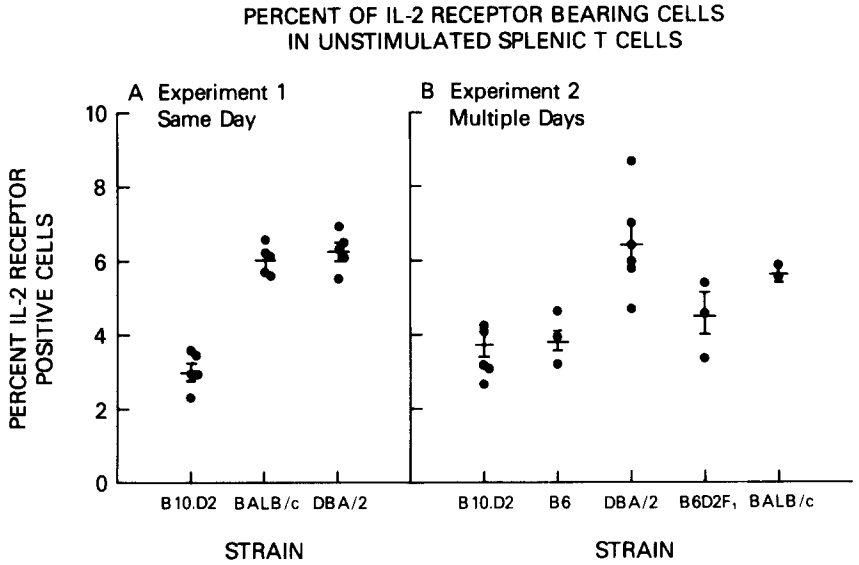

FIGURE 4. Genetic regulation of IL-2-R-bearing cells in unstimulated splenic T cells. (*A*) Nylon wool–passed spleen cells from unimmunized mice of strains B10.D2, BALB/c, or DBA/2 were labeled for IL-2-R by using 7D4 and FITC-MAR 18.5. Cells incubated with FITC-MAR 18.5 alone served as background control. Percent of positive cells was calculated as described in Materials and Methods. Five mice of each strain were tested individually. Means and SEM are indicated by horizontal lines. (*B*) Nylon-passed spleen cells of individual mice of strain B10.D2, B6, DBA/2, B6D2F_1, or BALB/c were tested as in *A*, except that within each strain, the individual animals were all tested on different days as controls for the experiment of Fig. 6.

(Fig. 4*B*). The strain differences were also consistent among mice from different shipments. No correlation of the observed differences with age (in the 8–20-wk range) or sex was noted (data not shown). It is also of interest that the F_1 hybrid between B6 (low) and DBA/2 (high) was intermediate in the percent of receptor$^+$ cells.

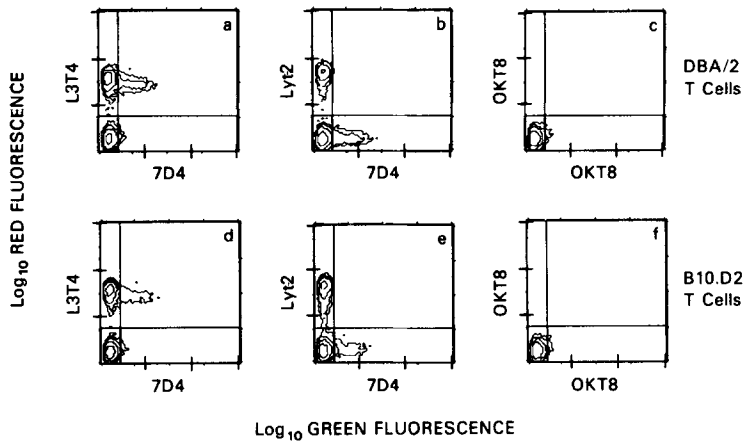

FIGURE 5. Two-color IF contour diagrams of DBA/2 (*a–c*) and B10.D2 (*d–f*) T cells. Cells were stained with FITC-7D4 (*a*, *b*, *d*, and *e*) or FITC-OKT8 (*c* and *f*) followed by biotinylated anti-L3T4 (*a* and *d*), or anti-Lyt2 (*b* and *e*) or OKT8 (*c* and *f*) followed by TRA (*a–f*). The percentage of cells in the four quadrants were as follows. Lower left (double negatives): *a*, 37.3%; *b*, 70.8%; *c*, 98.6%; *d*, 52.1%; *e*, 62.5%; *f*, 98.4%. Upper right (double positives): *a*, 5.5%; *b*, 0.55%; *c*, 0.37%; *d*, 2.93%; *e*, 0.73%; *f*, 0.52%. Upper left (cells positive for L3T4 or Lyt-2 but negative for 7D4): *a*, 55.6%; *b*, 22.4%; *c*, 0.27%; *d*, 43.9%; *e*, 33.2%; *f*, 0.24%. Lower right (cells positive for 7D4 but negative for L3T4 or Lyt-2): *a*, 1.17%; *b*, 5.9%; *c*, 0.75%; *d*, 0.70%; *e*, 3.0%; *f*, 0.86%. The channel numbers used to delineate positive and negative were chosen as described in Materials and Methods, using OKT8 reagents as negative controls.

The IL-2-R$^+$ Cells in Unstimulated Nonimmune Spleen Cells Are Found Only in the L3T4$^+$Lyt-2$^-$ Subpopulation. The distribution of IL-2-R$^+$ cells in unstimulated splenic T cells from unimmunized mice was determined by two-color FCM, using fluorescein-labeled 7D4 and biotin-coupled monoclonal anti-L3T4 or anti-Lyt-2, followed by TRA. Anti-OKT8, which does not react with murine cells (25, 26), was used as a negative control. The results, presented as contour diagrams in Fig. 5, were quite striking. All of the detectable 7D4-staining (IL-2-R$^+$) cells were in the L3T4$^+$ population and in the Lyt-2$^-$ population. This was confirmed by integration of the two-color data (Fig. 5). For each of the three strains tested, B10.D2, BALB/c (not shown), and DBA/2, neither the Lyt-2$^+$ population nor the L3T4$^-$ population of T cells ever showed more positive cells with FITC-7D4 than with the control FITC-OKT8. This contrasts with studies of T cells stimulated in vitro in which both L3T4$^-$Lyt-2$^+$ and L3T4$^+$Lyt2$^-$ subpopulations express IL-2-R (9), as well as with Con A–stimulated controls in our experiment. When we ran two-color FCM studies of Con A–stimulated spleen cells in parallel with unstimulated cells, ~83% of the Lyt-2$^+$ cells in the Con A–stimulated population were 7D4 staining, and Lyt-2$^+$7D4$^+$ cells accounted for 29% of the Thy-1$^+$7D4$^+$ Con A–stimulated spleen cells. The results for unstimulated T cells also contrast with observations of unstimulated thymocytes, among which IL-2-R-bearing cells are all L3T4$^-$Lyt-2$^-$ (10, 11). It is also apparent from the contour lines and computer integrations that the fraction of receptor$^+$ cells is nearly twofold greater in the DBA/2 than in the B10.D2 T cells, in either the L3T4$^+$ or the Lyt-2$^-$ populations. Thus, the observed difference is not due to a difference

between strains in the T cell subsets that express IL-2-R. The data for BALB/c were nearly identical to those for DBA/2 (not shown).

In control two-color FCM studies run in parallel with similar experiments, the following observations were made: (*a*) The majority of 7D4-staining (IL-2-R-bearing) cells in unstimulated nylon-passed splenic T cells were Thy-1^+, and Thy-1^+7D4^+ cells accounted for >90% of the $L3T4^+7D4^+$ cells. Thus, the IL-2-R-bearing cells could not be accounted for by contamination with non–T cells. (*b*) The fraction of $L3T4^+$ cells bearing IL-2-R in the fresh, nylon-passed splenic T cell populations did not decrease after these cells were put in resting culture without stimulants for 48 h. (*c*) In unseparated, unstimulated, fresh spleen cell populations, a similar fraction of Thy-1^+ cells bore IL-2-R (i.e., Thy-1^+7D4^+/Thy-$1^+ \cong 10\%$). However, as only 23.5% of the spleen cells were Thy-1^+, the $7D4^+Thy1^+$ population was only 2.5% of the total cells. Thus, this population would be harder to detect without T cell enrichment as above. Within limits of experimental error, these Thy-1^+7D4^+ cells were virtually all $L3T4^+Lyt\text{-}2^-$, as we had seen for the nylon-passed cells. Thus, the results are not due to artifacts of nylon wool purification of T cells. (*d*) In unseparated spleen cells, there was also a population of IL-2-R-bearing cells that were Thy-1^-, i.e., non–T cells, possibly B cells that have previously been shown to react with 7D4 in the mouse (12). This background of non–T cells made it difficult to study accurately T cells in unseparated spleen cells, so all further studies were performed on nylon-passed T cells.

RI Strains Indicate Multigenic Control But Suggest Mapping of at Least One Gene to Chromosome 7 That Influences the Level of IL-2-R$^+$ Cells. As the three strains compared (DBA/2, B10.D2, and BALB/c) are all *H-2*^{d}, the difference did not reside in the H-2 complex. Also, as BALB/c resembled DBA/2 in IL-2-R expression, but resembled B10.D2 in being *mls*^{b} (low) (27), the difference did not appear to be related to *mls* itself. Results below will confirm absence of linkage to either of these loci. As we were trying to map a gene that differed between the non-H-2 "background" genome of B10 congenics and that of DBA/2, we were fortunate to have available 23 B × D RI strains from the cross of B6 × DBA/2 (28). The B6 strain is nearly identical to B10 and is shown to have a low level of IL-2-R-bearing cells (Fig. 4*B*), almost identical to that of B10.D2. The RI strains were derived by inbreeding an F_2 generation of this cross to produce a series of inbred strains, each of which resembles an F_2 mouse, in that the original B6 and DBA/2 parental chromosomes are arbitrarily reassorted, but is expected to be homozygous at virtually every locus. The B × D RI strains had been typed at 73 genetic loci (reference 28 and B. A. Taylor, personal communication). Due to limited availability, we could test only two or three mice of each of 23 of the B × D RI strains (Fig. 6). Each strain was tested on more than one day and the reproducibility was satisfactory. The distribution of RI strains (Fig. 6) indicates multigenic control. However, it was striking, on inspection of the 73 known loci for which these strains had been typed, that the locus *HBB* on chromosome 7 segregated almost perfectly with the level of receptor-bearing cells. With only one exception (discordance) in 23 strains, the strains inheriting *HBB* from the B6 parent were at the lower end of the scale and those inheriting *HBB* from the DBA/2 parent were at the higher end of the scale (Fig.

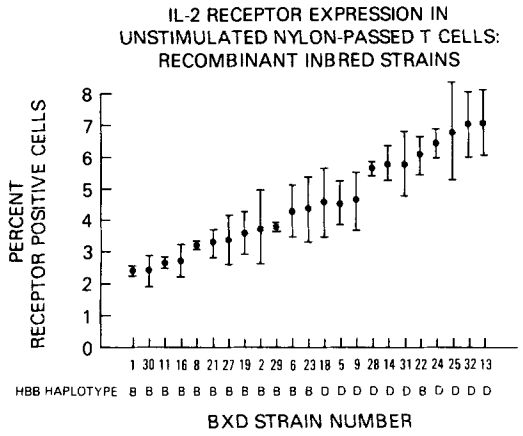

FIGURE 6. Fraction of IL-2-R-bearing cells in fresh, unstimulated splenic T cells from unimmunized mice of 23 B × D RI strains. Analysis was performed as in Figs. 3 and 4 on two to three mice of each strain. Each strain was tested on more than one day, in parallel with the controls in Fig. 4*B*. The haplotype of origin of the *HBB* gene (B for B6 and D for DBA/2) is indicated for each of the RI strains. Error bars are SEM.

6). This observation suggested that one of the genes involved might be closely linked to, but not identical with, the *HBB* gene. However, the involvement of multiple genes made it necessary to introduce a novel statistical approach to the problem to put this observation on a firm quantitative footing.

If a single gene regulating the level of receptor-bearing T cells in the DBA/2 and the B6 mouse were closely linked to an identified genetic locus, then it would be expected that a B × D RI strain bearing the marker D (DBA/2 origin) at this locus would tend to have a higher number of receptor-bearing cells than an RI strain having the marker B (B6 origin) at this locus. Thus, if such RI strains could be classified as high or low according to the number of receptor$^+$ cells, linkage could be determined. The 23 strains that we tested failed to show any cutoff value separating the strains into "high" and "low" classes (Fig. 6). We therefore looked for other evidence of linkage as follows. The strains classified as B at a given locus were collected and the corresponding percentages of positive cells were expressed as a cumulative distribution; the same was done for strains classified as D. The maximum vertical distance attained by the D-distribution over the B-distribution was then taken as a measure of linkage (one-sided Smirnov two-sample statistic; see Fig. 7). Clearly, this distance would be greatest if each of the strains classified as B at the locus in question had a lower percent of receptor-bearing cells than any of the strains classified as D (see Fig. 7*A*). On the other hand, if the two groups of percents overlapped extensively (i.e., were thoroughly mixed), the distance between the two distributions would be small (see Fig. 7*C*). We chose the one-sided statistic (29) as a measure of linkage because of the ordering of the percentages of receptor-bearing T cells in the parental strains (B<D). The foregoing method was applied to the data pertaining to each of the 73 loci. The levels of significance of the three Smirnov statistics shown in Fig. 7 are (using reference 30) 0.000019 for *HBB*, 0.04 for *MUP-1*, and >0.50 for *mls*. This is a nonparametric method that compares two distributions without any assumptions about the shape of the distributions (29).

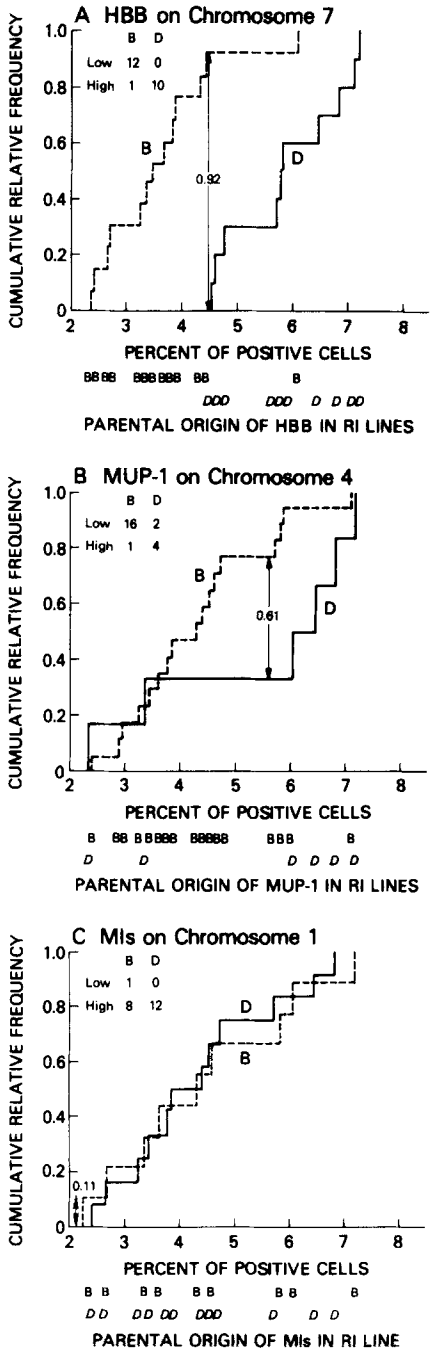

FIGURE 7. Cumulative relative frequency distributions of B × D strains bearing B or D alleles at three of the 73 loci tested, used to determine Smirnov statistics (vertical arrow) testing for possible linkage of that locus with the gene(s) regulating the fraction of IL-2-R-bearing T cells. Inset in each panel shows the optimum 2 × 2 table for that locus (minimum discordancies between high and low levels of receptor$^+$ cells and D and B alleles at that locus).

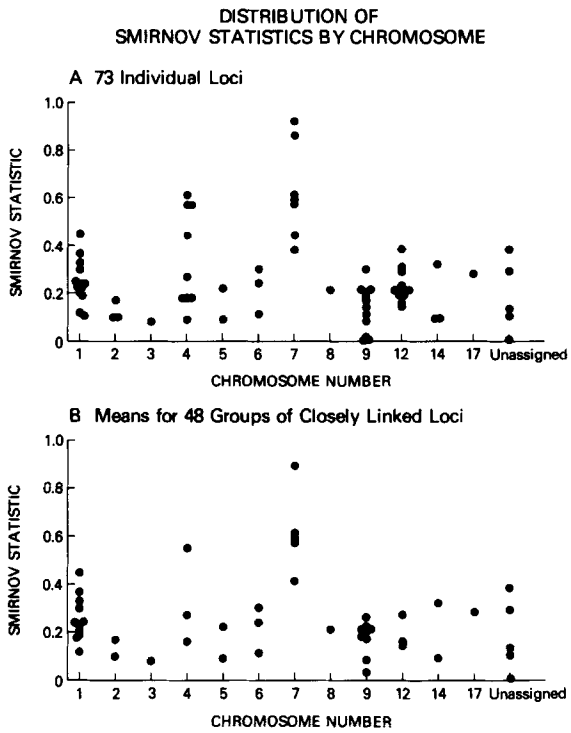

FIGURE 8. Distribution of Smirnov statistics by chromosome. (*A*) All 73 individual loci tested. (*B*) Means of Smirnov statistics for 48 groups of closely linked loci.

Although the individual Smirnov statistics and associated levels of significance are informative per se, considerably more importance is attached to the distribution of these statistics with respect to the various chromosomes. Values of the Smirnov statistics for all 73 markers, grouped by chromosome, are plotted in Fig. 8*A*. The statistics for two closely linked markers on chromosome 7, *HBB* and *MOD-2*, have values of 0.92 and 0.86, which stand out from all the rest. Moreover, the next three greatest Smirnov statistics associated with chromosome 7 loci [*MTV-1* (0.61), *TAM-1* (0.59), and *GPI-1* (0.57)] are equaled only by three tightly linked markers on chromosome 4 [*LYB-2* (0.57), *LYB-6* (0.57), and *MUP-1* (0.61)]. To minimize the influence of linkage on the process of comparing the Smirnov statistics, we grouped together all markers known to be within 10 cM of one another on the same chromosome (31). The Smirnov statistics in each group of linked markers were then averaged, and, as a result, 48 "independent" statistics were obtained. These values are shown in Fig. 8*B*. In this graph, four of the five highest points are associated with chromosome 7, and the remaining one with chromosome 4. It is apparent that none of the loci on chromosomes other than 4 or 7 is linked to the trait we are testing. Therefore, although we cannot determine with certainty the exact number of genes which influence IL-2-R expression in unstimulated splenic T cells, the data suggest that at least one gene of greatest influence maps on chromosome 7, near *HBB*, and that if there are other genes, their influence is more minor. One or more might map on

chromosome 4, although we cannot be sure, but there is no obvious linkage to any of the other chromosomes for which markers are available. From another point of view, the variability among the Smirnov statistics can be analyzed by comparing chromosomal averages of the values shown in Fig. 8*B*. The difference between the average for chromosome 7 (61.4%) and the overall average for the remaining chromosomes (21.1%) accounts for 86% of the variability among chromosomal averages ($p < 0.001$).

Thus, chromosome 7 is the only one to which a gene controlling the level of IL-2-R$^+$ cells can be mapped with the strains available. However, other genes play a role, and we cannot exclude the possibility of additional genes on chromosome 4 or on chromosomes lacking appropriate markers.

Discussion

In this paper we report several novel observations on IL-2-R expression. The first is that there is a small population of IL-2-R-bearing T cells in fresh, unstimulated spleen cells from unimmunized mice; moreover, these are exclusively in the L3T4$^+$Lyt-2$^-$ population, based on two-color fluorescence flow cytometry of purified T cell preparations. In contrast, in mitogen- or antigen-stimulated T cell populations, both L3T4$^+$Lyt-2$^-$ and L3T4$^-$Lyt-2$^+$ T cells express IL-2-R (9, 13, 32, and this paper). Second, there is a twofold difference in the fraction of unstimulated T cells bearing IL-2-R in spleens of unimmunized B10.D2 mice compared with those of DBA/2 or BALB/c mice, and this difference correlates with a comparable difference in responsiveness to IL-2. Previous studies suggested that unstimulated spleen cells should be unresponsive to physiologic concentrations of IL-2 (5–9 and 21–24). Our own results confirm this conclusion, but show that such cells do respond to much higher doses of IL-2 and that such responsiveness is genetically controlled. Third, this non-H-2-linked genetic difference in level of IL-2-R$^+$ cells is controlled by more than one gene, but at least one gene of major influence can be mapped to chromosome 7 by using RI strains. No linkage to the *mls* locus on chromosome 1 was found. The mechanism of action of the gene on chromosome 7 has not yet been determined. However, it does not influence the total number of L3T4$^+$ T cells, but only the fraction that bears IL-2-R.

IL-2-R-bearing T cells in fresh, unstimulated spleen populations from unimmunized mice have not been reported previously. It was unexpected that such cells should be exclusively L3T4$^+$Lyt-2$^-$, since it has been found that after mitogenic or antigenic stimulation in vitro, both Lyt2$^+$L3T4$^-$ and L3T4$^+$Lyt-2$^-$ cells express IL-2-R (9, 13, and 32). Indeed, L3T4$^+$Lyt2$^-$ cells appear to have more stringent requirements for optimal in vitro expression of IL-2-R than do Lyt-2$^+$L3T4$^-$ cells (9). Thus, the marked skewing of the population in unstimulated spleen cells suggests that the receptor-bearing cells we have observed are not merely due to low levels of the same type of stimuli previously shown to induce receptors in both L3T4$^+$ and Lyt-2$^+$ populations. In this regard, it is also of interest that a small (<3%) fraction of unstimulated thymocytes also has been shown to bear IL-2-R (10, 11). However, these cells were phenotyped as L3T4$^-$Lyt-2$^-$, and appear to represent a subpopulation of very immature T cells. In marked contrast, the unstimulated IL-2-R-bearing T cells reported here

exhibit a phenotype ($L3T4^+Lyt\text{-}2^-$) typical of a highly differentiated postthymic T cell.

The reason for the presence of IL-2-R^+ T cells in unprimed spleens is unknown. We have considered several possibilities. First, the mice we tested were clean and healthy, but not sterile, so that their immune systems could have been stimulated at some background level by environmental antigens and a very small fraction of T cells could have been activated to express IL-2-R continuously. However, even if this were the case, exogenous stimulation of the immune system should be a very common thing to which most of the mice are always exposed. There were not significant differences in the fraction of IL-2-R^+ cells among mice of the same strain from different shipments. Also, as mentioned, the stimuli would have to be selective for $L3T4^+$ cells. Finally, since the $receptor^+$ population does not decrease after two days in resting culture, continued environmental antigen stimulation is probably not required for receptor expression. For all these reasons, although genes may affect susceptibility to an environmental antigen, we think this explanation less likely.

Second, and more interestingly, these IL-2-R^+ cells might be autoreactive cells and be stimulated with autoantigen to express IL-2-R. Since there are many studies that show the presence of autoreactive T cells specific for H-2 or mls antigen, we tested the possible linkage between the fraction of IL-2-R^+ T cells and H-2 or mls antigens using RI B × D strains. There was no significant linkage (Figs. 7 and 8). Nevertheless, the most intriguing possibility is that these $L3T4^+$ IL-2-R-bearing cells are the cells responsible for an autologous MLR. In this case, the gene on chromosome 7 could encode another autoantigen important in the autologous MLR, or could influence sensitivity to the autologous MLR. These possibilities are currently being explored.

Third, these $L3T4^+$, IL-2-R^+ T cells could be different from usual T cells, and express IL-2-R without stimulation by antigens. Genetic differences could influence the size of this population. Fourth, all of the $L3T4^+$ T cells might have a variable but small number of IL-2-R, and we may be detecting the T cells with relatively high numbers of IL-2-R above a certain threshold. However, the shapes of the FCM curves (Fig. 3) do not support this possibility. The structural gene for the mouse IL-2-R has not yet been mapped.

To map the gene or genes influencing the level of IL-2-R^+ cells, we employed the statistical method of mapping RI strain data described above. The levels of $receptor^+$ cells among the 23 B × D strains tested did not fall into clear high and low groups and did not permit the determination of the number of genes involved, although the distribution indicated multigenic control. Nevertheless, we asked whether we could map any gene without having to define an arbitrary cutoff between high and low. We systematically examined all 73 known loci for all 23 strains to determine whether any of them appeared to be linked to the trait in question at any cutoff. The approach naturally lent itself to Smirnov statistics, which, to our knowledge, have not been used before for this purpose. For most loci, even optimizing the cutoff between high and low for that locus could not reveal any linkage. Moreover, we were able to show that, regardless of the number of genes involved, at least one of them of major importance appears to map on chromosome 7 near the *HBB* locus. Other unlinked genes are

probably also involved. A likely candidate site for a second gene is on chromosome 4, near *MUP-1*, but we cannot reliably map any genes to that site at this time. This approach may be of general usefulness in analyzing mapping data from RI lines.

Several alternative approaches were also studied. The percent of positive cells at which the maximum distance between two cumulative distributions occurs can also serve as a cutoff value, and the data pertaining to a given locus can be treated as a two-way classification of the individual strains: level of positive cells (high vs. low) compared with B vs. D origin of the locus. This classification provides an alternative measure of dissimilarity, the index of discordance, defined as the fraction of total strains with either a high number of receptors and locus of B origin or a low number of receptors and a locus of D origin. In Fig. 7, *A–C*, the indices of discordance are 1/23, 3/23, and 8/21, respectively. A third method of measuring dissimilarity is to choose the cutoff value so as to minimize the index of discordance. The distribution of the resulting minimized discordance has been studied by Gail and Green (33). In the three cases illustrated in Fig. 7, the minimum index of discordance occurs at the same point as the Smirnov statistic (maximum distance), with the indices noted above. None of the markers on chromosomes other than 7 have a minimum index of discordance as low as that of *HBB* on chromosome 7, no matter where the cutoff between high and low values is taken. Moreover, all other loci have many discordances at either the high end or the low end of the range or both, where the assignments to high and low are not ambiguous. Thus, although multiple genes probably influence the level of IL-2-R$^+$ T cells, the results strongly suggest that at least one major gene is on chromosome 7.

Summary

The present study reports the surprising observation that IL-2-R$^+$ cells can be detected in fresh, unstimulated, murine spleen T cells from unimmunized mice by flow cytometry using the monoclonal anti-receptor antibody 7D4. Also, unexpectedly, these cells were found exclusively in the L3T4$^+$Lyt-2$^-$ population by two-color fluorescence, in contrast to receptor$^+$ cells after stimulation, in which both L3T4$^+$Lyt-2$^-$ and Lyt-2$^+$L3T4$^-$ cells were found. The fraction of splenic T cells bearing IL-2-R reproducibly varies twofold under non-H-2-linked genetic control, with high expression in DBA/2 and BALB/c (~6–7%) and low expression in B10.D2 and C57BL/6 (3%). This correlates quantitatively with a greater responsiveness of the DBA/2 and BALB/c splenic T cells to high doses of IL-2, compared with B10.D2 T cells; twice as many B10.D2 T cells as DBA/2 T cells were required to get the same response. Studies with 23 B × D RI strains revealed that the level of IL-2-R$^+$ cells in unstimulated spleen cells was regulated by multiple genes, very likely including at least one gene on chromosome 7, near the *HBB* locus. The mapping makes novel use of nonparametric (Smirnov) statistics, which we suggest may be of general usefulness in similar analyses of RI strains.

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