SPECIFIC BINDING OF *K*- AND *I*-REGION PRODUCTS OF THE *H-2* COMPLEX TO ACTIVATED THYMUS-DERIVED (T) CELLS BELONGING TO DIFFERENT Ly SUBCLASSES

By ZOLTAN NAGY,* BRUCE E. ELLIOTT,‡ AND MARKUS NABHOLZ§

(From the Basel Institute for Immunology, Postfach 4005 Basel 5, Switzerland)

Mouse T cells activated against allogeneic cells in vitro after mixed leukocyte culture (1), or in vivo after transfer of T cells into irradiated *H-2* incompatible recipients (2), carry stimulator alloantigens on their surface. These surface-bound alloantigens are detected on the T-blast cells with alloantibodies directed against *H-2* determinants of the stimulator cells, followed by immunofluorescent staining with anti-mouse Ig reagents (1, 2).

Specificity of alloantigen binding by responder T blasts is suggested by the evidence that T cells activated in the mixed leukocyte reaction (MLR)[1] against a mixture of allogeneic cells carrying two different *H-2* haplotypes belong to two practically non-overlapping cell populations, each of which binds alloantigens derived from only one stimulator strain (1). Similarly, among T cells activated against stimulator cells incompatible for the whole *H-2* complex, at least two different antigen-binding cell populations can be distinguished, one of them carrying *K*-region and the other *I*-region stimulator cell products on their surface. However, in this experiment we observed a significant overlap (about 20%) of the two cell populations binding *K*- and *I*-region products, respectively. This overlap could be due either to the existence of multispecific cells or to the attachment, via specific binding of one type of product, of membrane fragments containing the other type. That the latter explanation is more likely is suggested by recent experiments demonstrating binding of subcellular antigen by MLR-activated blasts, from which antigen was removed by trypsin treatment.[2] One of the most effective sources of antigen was material from supernates of MLR cultures which could be sedimented by centrifugation at 100,000 *g*.

In the study reported here, we demonstrate specific binding of allogeneic *K*-

* Present address: Medical Research Institute, Hungarian Academy of Science, Budapest XIV, Hungary.

‡ Research fellow of the National Cancer Institute of Canada.

§ Present address: Swiss Institute for Experimental Cancer Research, 1011 Lausanne, Switzerland.

[1] *Abbreviations used in this paper*: B6, C57BL/6J; CTL, cytotoxic T cells; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; NMS, normal mouse serum; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine.

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and *I*-region products by responder T blasts, under conditions where these antigens are presented on separate stimulator cells. Induction of cytotoxic T cells (CTL) in allogeneic MLR depends largely on an incompatibility at the *K* (or *D*) region, and very likely the large majority of the CTL specifically recognize determinants on the *H-2K* and *H-2D* molecules themselves. *H-2I*-region incompatibility, on the other hand, gives in general a much stronger proliferative response than *K*-region incompatibility (3–5).

The demonstration by Shiku et al. (6) and Cantor and Boyse (7), that at least among cells from C57BL/6J (B6) mice and probably from the closely related C57BL/10 mice the cells responsible for these two types of responses express different T-cell-specific Ly alloantigens, gave the first substantial direct support that these functions were associated with distinct T-cell subclasses. The results of these investigators showed that CTL generated in allogeneic MLR cultures (and their precursors) belong to the Ly $1^-2^+3^+$ subclass; whereas most of the T cells responsive to *I*-region incompatible stimulators belong to the Ly $1^+2^-3^-$ subclass (7). The present experiments strongly suggest that T cells binding *I*-region and *K*-region products, respectively, correspond to the functional subclasses of Ly $1^+2^-3^-$ and Ly $1^-2^+3^+$ cells described by Cantor and Boyse (7).

Materials and Methods

Mice. A/J mice (Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland), B6, C3H/J, and SJL mice (Bomholtgaard, Ry, Denmark) and recombinant mouse strains A.TH, A.AL, and A.TL (a gift from Dr. D. C. Shreffler, Washington University, St. Louis, Mo.) were used. F_1 animals were produced in our own facilities.

Media. Medium used for tissue culture was HEPES-buffered (10 mM) RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with L-glutamine, streptomycin, penicillin, 5% human serum, and 2-mercaptoethanol as described previously (1). The same medium without 2-mercaptoethanol and containing only 2.5% human serum was used for washing cells.

MLR. Unidirectional MLR cultures were set up using X-irradiated (3,300 R) lymph node cells or lymph node plus spleen cells as stimulator cells. The responder cells were nylon wool column-passaged (8) lymph node cells [enriched for T cells with 0.7–2.7% B (Ig^+) cell contamination]. The concentration of responder cells was adjusted to 5×10^6/ml, and that of each stimulator cell population to 1×10^7/ml. Cultures were set up in 30-ml plastic flasks (catalogue no. 3012; Falcon Plastics Div. of BioQuest, Oxnard, Calif.) and incubated in an upright position at 37°C in a 5% CO_2-95% air, water-saturated atmosphere for 4 days. For each culture, 10^7 responder cells were mixed with 2×10^7 of each stimulator cell type, except for two experiments in which 4×10^7 (A.TH × A.TL)F_1 instead of A.TH stimulator cells were used. [In our system twice as many (A.TH × A.TL)F_1 cells as A.TH stimulator cells yielded comparable degrees of stimulation.] MLR activation was assessed by measuring incorporation of [^{3}H]thymidine or [^{125}I]uridine (after a 4 h pulse) in 200-μl aliquots of the cultures at the time of harvest (9).

Antisera. Anti-Ly-1.2 (C3H/An anti-CE/J thymocytes) and anti-Ly-2.2 [(C3H/An × C57BL/6-Ly-2.1)F_1 anti-ERLD (radiation-induced leukemia from B6)] sera (10) were a gift from Dr. H. Cantor, Division of Tumor Immunology, Sidney Farber Cancer Center, Boston, Mass. These sera had previously been shown to react specifically with the Ly-1 and Ly-2 antigens, respectively, controlled by the Ly alleles present in the B6 strain (7, 11). Before use, the Ly antisera were diluted 1:10 in phosphate-buffered saline (PBS) and absorbed with 3×10^8 C3H thymus plus lymph node cells/ml to remove autoantibodies.

Hyperimmune A.TL anti-A.TH and A.TL anti-A.AL sera were a gift of Dr. D. C. Shreffler, Washington University. 10 volumes of antisera were absorbed with 7 volumes of packed spleen and lymph node cells (as described in Fig. 1) two times at 4°C for 20 min. All alloantisera were heat inactivated (56°C, 30 min), aliquoted and stored at −70°C until use.

Rabbit anti-mouse T serum (12) was a gift from Dr. C. Bron, Institut de Biochimie, Université de Lausanne, Switzerland. Tetramethylrhodamine [(TRITC)]-sheep anti-rabbit Ig and fluorescein

isothiocyanate [(FITC)]-sheep anti-mouse Ig were kindly provided by L. Forni, Basel Institute for Immunology.

Complement. The source of complement (C) was pooled normal rabbit serum absorbed as described by Boyse et al. (13), except that an additional absorption step with agarose (0.3% wt/vol, 4°C, 30 min) was included.

Treatment of MLR-Activated Cells with Anti-Ly Sera and C. Viable cells were recovered from MLR cultures by centrifugation over a Ficoll-Urovison layer (density 1.09 g/cm^3) at 2,000 *g* for 15 min. 30–50% of the viable cells thus obtained were large or medium sized blasts. After three washes in Hanks' balanced salt solution (HBSS; Flow Laboratories, Glasgow, U.K.) containing 2 or 5% heat-inactivated fetal calf serum (FCS), cell suspensions were adjusted to 4×10^7/ml.

On the basis of preliminary experiments, the following general procedure for anti-Ly treatment of MLR-activated cells was adopted: One volume of cell suspension (in 2% FCS) was mixed with one volume of the appropriate anti-Ly serum or normal mouse serum (NMS) diluted 1:15 in PBS, and the mixture incubated at 4°C for 30 min. Then, one volume of freshly thawed rabbit C (diluted 1:4 in PBS) was added without further washing, and incubation at 37°C continued for 45 min. We used, for each antiserum, the concentration of C that was optimal when the cytolytic antiserum activity against (B6 × A.TL)F_1 blasts was assessed in a microcytotoxicity dye exclusion assay.

Immediately after incubation with C, 10 μl of the cell suspension were removed and the percentage of blasts and total cells still able to exclude Eosin Y (1% in PBS) was determined. The remaining sample was washed with cold HBSS plus 5% FCS.

In each of the three experiments reported in Fig. 2 the treatment with anti-Ly sera differed in the following way: In exp. 1, cells were treated once; in exp. 2, twice; and in exp. 3, three times with sera plus C. After the final treatment, viable cells were recovered by centrifugation on Ficoll-Urovison, before staining with anti-stimulator alloantisera.

In all experiments the cytotoxic effect of the treatment with anti-Ly sera was monitored by dye exclusion assays in aliquots of the treated T-cell blast populations. Specific killing (compared with NMS and C) of MLR-activated (B6 × A.TL)F_1 blasts treated with either anti-Ly serum and C ranged between 23.8 and 35.6%, and appeared to increase with the number of treatments. Lysis in the groups treated with NMS plus C ranged between 13 and 25%. No specific killing of SJL-activated C3H blasts with anti-Ly sera was observed; thus no autoantibodies against C3H in the sera were detected.

Treatment with both anti-Ly-1 and anti-Ly-2 serum plus C did not give significant additive effects in terms of cytotoxicity measured by dye exclusion. On the other hand, slides prepared from such doubly treated cell populations, contained very few cells which would be acceptable for scoring, i.e. cells that were morphologically intact (as assessed by phase-contrast illumination), and that showed a characteristic continuous pattern of fluorescence observed with rabbit anti-T antibodies detected by (TRITC)-sheep anti-rabbit Ig (1). We concluded that, although the anti-Ly treatments were insufficient to kill all cells expressing the respective Ly specificity when killing was assessed by dye exclusion, all or most of these cells were sufficiently damaged to be excluded when the ethanol-fixed preparations were scored for antigen-binding cells.

Incubation of Cells with Alloantisera and Immunofluorescence. Detailed procedures have been described previously (1). Briefly, cells were incubated with different dilutions of alloantisera at 4°C for 45 min. After three washes in HBSS (with 10 mM HEPES, 5% FCS, and 10 mM sodium azide) immunofluorescent double staining was performed. The surface-bound alloantibodies were visualized by direct immunofluorescence with (FITC)-sheep anti-mouse Ig. The same cells were exposed to rabbit anti-mouse T serum and (TRITC)-sheep anti-rabbit Ig to verify the presence of T-cell markers on the antigen-binding cells.

Percentages of T^+Ig^+ blasts were calculated from cell counts obtained by scoring at least 200 blasts in one or two preparations per group. The average deviation between two slides from the same group was 5.3 % (extremes 0.4 and 15.3%) when scored by the same or two different investigators.

Results

Rational of the Experiments. The choice of the responder-stimulator combinations for the experiments reported here was based on two considerations:

(*a*) In order to avoid complications due to membrane fragments containing both *K*- and *I*-region

products, we chose a responder-stimulator combination in which *K*- and *I*-region determinants could be presented on separate stimulator cells. In this combination the responder strain carries a haplotype derived from a crossover between the *K* and the *I* region, and the stimulators were the two parental strains. The antisera used to detect stimulator antigens had been previously characterized only by cytotoxicity (14). They were therefore absorbed as described in Fig. 1 before use in immunofluorescence studies. This experimental design obviated any complications due to possible cross-reactions at the level of T-cell recognition or antisera.

(*b*) Extrapolation of the classification of functionally differentiated T-cell subsets on the basis of their Ly phenotype (7, 15) to cells from mouse strains other than B6 mice and closely related strains typed for their Ly phenotype, has in some cases been difficult (6). Furthermore, although strain A (which is congenic with A.TL) carries the same Ly antigens as B6 (16), the Ly-antigen specificities expressed by strain A.TL have not been confirmed by direct typing. Thus, we decided to include the B6 genome in the responder cells making the assumption that the (B6 × A.TL)F_1 cells would behave like B6 with regard to Ly-antigen expression. [Preliminary experiments indicated that the proportions of normal (B6 × A.TL)F_1 lymph node cells with Ly-1 or Ly-2 surface antigens were similar to those of B6 lymph node cells as determined by a dye exclusion microcytotoxicity test (unpublished results)].

Binding of K- or I-Region Products by MLR-Activated T Blasts. To investigate whether stimulator antigens controlled by genes in the *K* and *I* regions of the *H-2* complex, are bound by different T blast populations, nonadherent lymph node cells from (B6 × A.TL)F_1 mice (*H-2* haplotypes: $K^bI^bS^bD^b \times K^sI^kS^kD^d$ = *bbbb* × *skkd*) were activated against both strain A.TH (*sssd*) and A.AL (*kkkd*) cells in the same culture. Thus the responder cells differed from A.TH at the *I* and *S* region, and from A.AL at the *K* region only.

Of the responder blasts, 51.4% could be stained with anti-I^s serum and 43.8% with anti-K^k serum at serum dilutions of 1:20 (Fig. 1). The fraction of binding cells reached almost a plateau at dilutions of 1/40 for either alloantiserum. After incubation with both sera together, the proportion of T^+Ig^+ blasts was 88.5% at final sera dilutions of 1:20 and 83.9% at dilutions at 1:40. These values are very close to the sum of the percentages of T^+Ig^+ cells observed when each serum was administered to separate aliquots of responder blasts. An average of only 5% of (B6 × A.TL)F_1 cells, activated against A.TH, bound detectable amounts of anti-K^k serum (Fig. 1). It is impossible to obtain a comparable background figure for anti-I^s serum, since (B6 × A.TL)F_1 does not respond significantly in MLR to A.AL (5). However, the specificity of staining wtih anti-I^s serum was confirmed in previous experiments, in which B6 responder cells stimulated with A.AL cells were stained with anti-I^k serum but not with anti-I^s serum (3). These results strongly suggest that in these cultures each activated responder cell carries receptors specific for either *K*-region or *I*-region products of the stimulator cells but not both.

The Ly Phenotype of T Blasts Binding K- and I-Region Antigens. The results of three experiments in which the effect of treatment with anti-Ly-1 or anti-Ly-2 serum and C on antigen-binding T blasts was tested, are shown in Fig. 2. Treatment with normal mouse serum and C (Fig. 2, upper panel) does not significantly change the proportions of T blasts which bind *K*- and *I*-region products observed without anti-Ly serum treatment. (Data for these control experiments were similar to those in Fig. 1 although the percentages of cells stained by each antiserum were somewhat lower.) Anti-Ly-1 serum eliminates virtually all cells that could be stained with antiserum directed against stimulator *I*-region determinants (Fig. 2, middle panel). At the same time, the fre-

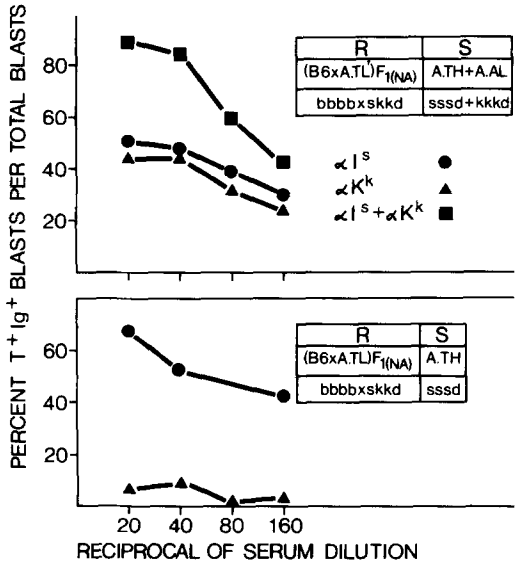

FIG. 1. Nylon wool-passaged nonadherent (NA) (B6 × A.TL)F_1 lymph node cells (1.9% Ig^+) were activated in MLR against A.TH or a mixture of both A.TH and A.AL stimulators (S). The resulting blasts were stained with different dilutions of anti(α)-I^s serum or anti-K^k serum, or both together. αI^s: A.TL anti-A.TH absorbed (10 volumes) with (B6 × A.TL)F_1 (5 volumes) and A/J (2 volumes) cells. αK^k: A.TL anti-A.AL absorbed (10 volumes) with (B6 × A.TL)F_1 (5 volumes) and A.TH (2 volumes) cells. R, responders.

quency of cells binding stimulator *K*-region determinants increases to the level of the proportion of cells stained with both antisera together. Treatment with anti-Ly-2, serum gives the reverse effect (Fig. 2, lower panel); the proportion of cells detectable by staining with antiserum against stimulator *K*-region determinants is reduced to background levels and the percentage of cells binding stimulator *I*-region determinants increases. Repeated treatments with antiserum and C as in exps. 1–3 (Fig. 2) resulted in increased depletion or enrichment of the corresponding populations.

Discussion

The suggestion that T cells responsive to major histocompatibility complex (MHC) determinants belong to two functionally differentiated subclasses had been based primarily on the premise that T cells conform with the tenets of the clonal selection theory, and on the experimental findings that in the course of an MLR, stimulation of the proliferative response measured by [^{3}H]thymidine uptake and the induction of measurable specific CTL activity depended on genetically separable allogeneic determinants (3–5). Recent experiments by Shiku et al. (6) and Cantor and Boyse (7, 15) have directly shown that indeed CTL and their precursors (6, 7, 15) on the one hand, and the cells on which a strong proliferative response to *I*-region incompatible stimulators depends (7, 15) on the other, represent two subclasses of T cells which express Ly $1^-2^+3^+$ and Ly $1^+2^-3^-$ phenotypes, respectively.

The results in Fig. 1 provide a direct demonstration that among T blasts activated against a mixture of *K*- and *I*-region incompatible stimulators the

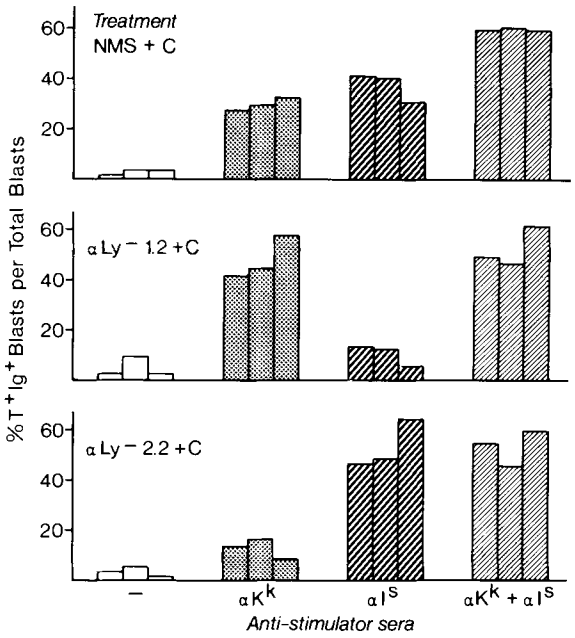

FIG. 2. Nylon wool passaged nonadherent (NA) (B6 × A.TL)F_1 lymph node cells (0.7–2.7% Ig^+) were activated in MLR against a mixture of both A/J and A.TH [or (A.TH × A.TL)F_1] spleen and lymph node cells. The results of three separate experiments are listed in which cells were treated once, twice, or three times, respectively, with NMS or anti(α)-Ly-1.2 or anti-Ly-2.2 sera and C. Surviving blasts in each group were incubated alone or with anti-K^k serum or anti-I^s serum, or both (absorbed as described in Fig. 1) at a final dilution of 1:20. The percent T^+Ig^+ blasts was determined by immunofluorescence as described in the text. The stimulation indices were 87.3, 83.7, and 115.6 in exps. 1, 2, and 3, respectively.

populations binding *K*-region and *I*-region stimulator determinants are most or completely non-overlapping. Furthermore, as the results in Fig. 2 indicate, all or almost all cells binding *K*-region products express Ly-2 but not Ly-1 antigens, while the cells binding *I*-region products belong to an Ly 1^+2^- subclass. Although we did not test the effects of an anti-Ly-3 serum, we assume in the following discussion, on the basis of the work of Shiku et al. (6) and Cantor and Boyse (7), that this would give the same results as treatment with anti-Ly-2 sera.

In the population of responder T cells activated against *K*- and *I*-region determinants almost half the T blasts bind *K*-stimulator determinants. This is consistent with an experiment by Cantor and Boyse who tested the effect of pretreatment with anti-Ly sera and C on the magnitude of [^{3}H]thymidine incorporation in *I*-region and (*I* + *K*)-region incompatible cell mixtures (7). It is known that in the very same combinations used by Boyse and Cantor, *K*-region incompatibility alone gives at best a marginal specific increase in [^{3}H]thymidine uptake (17). Therefore, *K*-region-responsive cells probably contribute a significant proportion of the total thymidine incorporation after activation in presence of an ongoing response to *I*-region incompatibility. This interpretation is, of course, consistent with the finding that in some culture

systems a CTL response against *K*-region determinants is strongly enhanced when it is accompanied by a response to *I*-region incompatibilities.[3]

What has yet to be demonstrated is that the precursors of T blasts binding *I*-region and *K*-region stimulator determinants belong to the Ly $1^+2^-3^-$ and the Ly $1^-2^+3^+$ T-cell subclasses, respectively; and whether among unstimulated T cells there is an association between *I*- and *K*-region specificity (in addition to responsiveness) and Ly phenotype. Treatment of the precursor population with anti-Ly-1 or anti-Ly-2 serum and C would almost certainly prevent appearance of the *I*- or the *K*-region antigen-binding cells in the activated cell population. To establish, however, that all *I*-region- and all *K*-region-specific precursor cells belong to the Ly $1^+2^-3^-$ and the Ly $1^-2^+3^+$ subclasses requires demonstration of MHC antigen binding by the precursors. In our assay, we have not yet been able to detect specific MHC antigen binding by unstimulated small lymphocytes.

One of the most puzzling questions about T-cell immune responsiveness concerns the significance of the high proportion of cells reactive to allogeneic determinants controlled by genes of the MHC. The demonstration, that among MHC-reactive T cells responsiveness to different types of determinants is correlated with differences not only in functional activity but also in the expression of cell surface Ly antigens, supports the belief that it is the biological function of MHC products to be recognized by lymphocytes and that the immunologically detectable variability of these molecules is the result of evolutionary adaptation. From this point of view it seems likely that the association between Ly phenotype and functional potential of T cells is a reflection of the function of the molecules bearing the Ly determinants themselves. But the differentiation of T cells reactive to different MHC incompatibilities raises also some intriguing questions about the ontogeny of T-cell specificity.

There seem to be two types of explanations for the association of T-cell subclass and specificity: (*a*) Firstly, it is possible that among mature T cells as many Ly $1^+2^-3^-$ cells express receptors for *K*-region determinants as Ly $1^-2^+3^+$ cells, and vice versa for *I*-region determinants, but that the two cell types differ in their requirements for activation. Of the cells specific for a particular *K*-region determinant only the Ly $1^-2^+3^+$ cells could respond to the specific stimulus, while the converse would hold for *I*-region-specific cells. (*b*) Alternative explanations imply that the receptor dictionaries of the two T-cell subclasses are different, i.e. that during ontogeny commitment to expression of a receptor of one type of specificity entails acquisition of the corresponding Ly phenotype at some stage of maturation. This association might reflect the existence of two classes of genes coding for T-cell receptor specificity, whose respective expression is, at the level of the genome, coupled with that of genes coding for different Ly antigens and endowing the cell with different functional properties. But there also exists the possibility that in the course of a somatic

[3] One point which requires comment is that in other culture conditions the magnitude of the CTL response is independent of *I*-region incompatibility between responder and stimulator (5). The culture system and the responder cell preparation used in the experiments reported now were designed to minimize activation due to nonspecific stimuli, such as FCS, and we have indeed found that in this system the CTL response of (B6 × A.TH)F_1 cells to stimulation by A cells alone is more than eightfold lower than to a mixture of A and A.TH stimulators (unpublished results).

generation (or restriction) of diversity, cells becoming committed to specificity for *K*- or *I*-region determinants are "imprinted" to express, at some stage, different Ly phenotypes and functional potentials. This could occur as a result induced by the recognition of self-determinants, a first step in one of the possible mechanisms for somatic generation of T-cell diversity, originally suggested by Jerne (18) and recently discussed by one of us (19). The subclass to which a T cell belongs as well as the type of specificity of its receptor could be determined by the type of self-antigen with which its self-reactive ancestor interacted.

Summary

Responder cells [C57BL/6J × A.TL)F_1 lymph node cells depleted of bursa equivalent-derived (B) cells by filtration through nylon wool columns] were activated against incompatible *K*-region and *I*-region products together under conditions where these antigens are presented on separate stimulator cells. The resulting T blasts were stained with different concentrations of antisera directed against incompatible stimulator *K*-region or *I*-region products, or both. We obtained results that strongly suggest that in these cultures each activated responder blast stains with antiserum directed against either *K*-region or *I*-region products, but not both.

Responder blasts from the same cultures were treated with antiserum and complement (C) directed against either Ly-1.2 or Ly-2.2 T-cell-specific surface antigens. Anti-Ly-1.2 serum and C specifically eliminates virtually all responder blasts staining with antiserum directed against stimulator *I*-region products; whereas anti-Ly-2.2 serum reduces to background levels the proportion of cells staining with antiserum against stimulator *K*-region products.

The results obtained suggest that T cells binding stimulator *K*-region and *I*-region products, respectively, belong to two different subclasses distinguishable by their Ly phenotypes. Possible explanations for this association of T-cell subclass and specificity are discussed.

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