T-T INTERACTIONS IN THE INDUCTION OF SUPPRESSOR AND HELPER T CELLS: ANALYSIS OF MEMBRANE PHENOTYPE OF PRECURSOR AND AMPLIFIER CELLS

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It is now clear that the outcome of exposure of an animal to antigen is governed by not only the route, nature, and dose of antigen, but also by the subsequent series of cell interactions which occur. Analysis of these interactions has been facilitated by the use of antisera to lymphocyte differentiation antigens and in the case of T lymphocytes, the Ly (1) series of alloantigens has been particularly useful (2–5). Experiments with these antisera have allowed more definite separation of T-cell subsets than had been possible previously. Furthermore, earlier data (6, 7) suggesting that interactions between T cells occur in the generation of T-killer cells in vitro, have been conclusively confirmed (4).

In the humoral response, in vivo data suggested involvement of more than one T-cell subset in helper function (8, 9). Our in vitro studies (10) have characterized the cells required as a long-lived adult thymectomy (ATx)[1]-resistant T_2 cell and a short-lived anti-lymphocyte serum (ALS)-resistant T_1 cell. We have also shown that a similar interaction occurs in the development of suppressor cells.

In this report we examine the surface membrane phenotype of the cells involved in these interactions and show that the precursors of helpers and suppressors have the same Ly phenotype as the differentiated effector cells. On the other hand, our data show that during differentiation of suppressor cells there is a change in expression of Ia antigens. Differentiation of helpers and suppressors is in each case aided by cells found in the T_1 pool. These 'amplifier' cells differ in surface phenotype from either the helpers or suppressors.

Materials and Methods

ATx. Mice were thymectomized at 6–8 wk of age (11) and used 16–24 wk later.

ALS. Rabbit anti-mouse thymocyte serum was raised by standard methods (12). It was kindly given by Dr. E. Simpson (Clinical Research Centre, Harrow, England). Mice were given 0.25 ml subcutaneously 2 days before sacrifice.

Antisera. Anti-Ly sera and anti-Ia^k were prepared as described previously (13, 14). Ly sera were tested for specificity on lymph node cells of antigen-positive and negative mice by a two-stage ^{51}Cr release assay. Sera showing any reactivity on the antigen-negative target were absorbed (30 min at room temperature with an equal volume of spleen, thymus, and lymph node cells) and retested. Anti-Ia^k was tested in a similar fashion on spleen cells after lysis of red blood cells. The

[1] *Abbreviations used in this paper*: ALS, anti-lymphocyte serum; ATx, adult thymectomy; KLH, keyhole limpet hemocyanin; TNP, trinitrophenol.

titers of sera used are given below (expressed as highest dilution giving maximal plateau lysis of appropriate target cells): anti-Ly-1.1 1:80–1:160; anti-Ly-2.1 1:8–1:32; anti-Ly-3.2 1:16–1:32; and anti-Ia^k (A.TH anti-A.TL) 1:100–1:512. The source of complement was rabbit serum selected for low toxicity and high complement activity and absorbed at a ratio of approximately 5:1 serum to cells with packed mouse lymphoid tissue. Absorbed complement was tested and generally used at a dilution of 1:10.

For bulk treatment of lymphocytes, 0.1 ml antiserum at a concentration giving maximal lysis was used per 10^6 cells. After 30-min incubation at 37°C, cells were washed once and resuspended in the same volume of complement. After a further 45-min incubation the cells were washed and the viable count determined by trypan blue dye exclusion.

Cell Culture. Techniques for the in vitro induction and assays for activity and specificity of helper and suppressor T cells have been described previously (15). The salient features of the methods are as follows. All cultures are carried out in Marbrook-Diener flasks (16). For induction of helper cells, approximately 15×10^6 spleen cells were cultured with a low dose, 0.1 μg/ml of keyhole limpet hemocyanin (KLH), a gift of Dr M. B. Rittenberg, Department of Microbiology, University of Portland Medical School. Helper cells were assayed in a second culture by their ability to cooperate with unprimed spleen cells in the presence of trinitrophenol (TNP) KLH in the induction of an IgM plaque-forming cell response to trinitrophenylated KLH (TNP KLH) (15).

Suppressor cells were induced in a similar fashion but with a high dose of antigen, 100 μg/ml KLH. Suppressor cells were assayed by their ability to abrogate the anti-TNP response of secondary cultures containing optimal numbers of in vitro induced helpers (17).

In most of the experiments to be described, helpers and suppressors were induced from 1:1 mixtures of spleen cells from ATx- and ALS-treated mice. Antigens, and assays for plaque-forming cells, were described previously (18).

Mice. CBA/Ca mice bred at the ICRF Animal Unit, Mill Hill, were used. Some mice were vaccinated (with vaccinia virus) at 4 wk of age and allowed to recover for approximately 8 wk before use.

Results

Phenotype of Helper Cell Precursor. Experiments using double chamber flasks have shown that the precursors of helper cells reside in the ATx spleen cell pool (10, Feldmann, manuscript in preparation). We were able, therefore, to examine the antigenic phenotype of this cell by treating ATx spleen (or T cells purified with nylon wool from ATx spleen) with antiserum and complement. The treated cells were mixed with an equal number of ALS spleen cells, and the mixture was cultured for 4 days with a low antigen dose to induce helper cells. These were assayed as usual in a second culture (15). The result of one such experiment is shown in Table I. It indicates that the helper cell precursors are $Ly\text{-}1^+2^-3^-Ia^-$. Three such experiments were performed.

Phenotype of Helper Cell Amplifier. It is known from double chamber experiments that the T cells of ALS spleens do not provide helper precursors, but are essential for maturation of $Ly\text{-}1^+$ helper precursors into effector cells. We have chosen to call these cells amplifiers by analogy with the cells that amplify the response of cytotoxic T-cell precursors to alloantigens (4).

The phenotype of helper amplifiers was investigated by treating ALS spleen cells with antiserum and complement and mixing the treated cells with equal numbers of viable ATx spleen cells before helper cell induction and assay. The results of one such experiment are shown in Table II. It indicates that the helper cell amplifiers are $Ly\text{-}1^-2^+3^+Ia^-$. This result was obtained in six experiments.

Phenotype of Suppressor Cell Precursors. Since suppressor cell precursors are also found in ATx spleen (19), experiments exactly analogous to those detailed above for helper cells were performed to examine the phenotype of

TABLE I
Membrane Phenotype of Helper Cell Precursor

Helper cells	Antigen	IgM response (AFC/culture ± SE)
–	DNP beads	753 ± 24
–	–	23 ± 12
–	TNP KLH	13 ± 9
Spleen	"	500 ± 64
ATx	"	20 ± 11
ALS	"	43 ± 23
ALS + ATx	"	513 ± 150
ALS + ATx (αLy-1 + C′)	"	40 ± 21
ALS + ATx (αLy-3 + C′)	"	473 ± 129
ALS + ATx (αIa^k + C′)	"	973 ± 299
ALS + ATx (C′)	"	680 ± 50

Helper cells were induced from CBA spleen, ATx, ALS, or 1:1 mixtures of treated or untreated cells. After 4 days culture 3×10^5 surviving 'helper' cells were assayed with 15×10^6 unprimed spleen cells and TNP KLH. This experiment indicates that the phenotype of helper cell precursors is Ly-1$^+$. Data from a single experiment (three flasks/group, arithmetic mean ± standard error of mean). Three such experiments were performed.

TABLE II
Membrane Phenotype of Helper Cell Amplifier

Helper cells cultured	Antigen	IgM response (AFC/culture ± SE)
–	–	20 ± 15
–	TNP KLH	53 ± 28
Spleen	"	520 ± 102
ALS	"	0
ATx	"	56 ± 14
ALS + ATx	"	417 ± 16
ALS (αLy-1 + C′) + ATx	"	57 ± 42
ALS (αLy-2 + C′) + ATx	"	30 ± 25
ALS (αLy-3 + C′) + ATx	"	0
ALS (αIa^k + C′) + ATx	"	306 ± 32
ALS (C′) + ATx	"	450 ± 6

Results from a single experiment. Six such experiments were performed. Phenotype of helper cell amplifier is Ly-1$^+$2$^+$3$^+$Ia$^-$.

suppressor cell precursors. Cells from ATx spleens were treated with antiserum and complement, mixed with equal numbers of viable ALS spleen cells, and induction and assay of suppressor cells carried out as described (17). Table III shows an experiment of this kind. In the majority of experiments (4/5) the suppressor precursor phenotype was found to be Ly-1$^-$2$^+$3$^+$Ia$^-$ (using anti-Ia^k at 1/10). In one experiment in which anti-Ia serum at a dilution of 1:10 was used, the precursors appeared to be susceptible to this antiserum. In a subsequent titration experiment no effect of anti-Ia was seen at dilutions from 1:5–1:100.

Phenotype of Suppressor Cell Amplifier. The phenotype of the cell resident in the ALS-treated spleen cells and required for suppressor cell induction was investigated by treating ALS spleen cells with antiserum and mixing the

TABLE III
Membrane Phenotype of Suppressor Cell Precursor

Helper cells	Suppressor cells	Antigen	IgM response (AFC/culture ± SE)
–	–	–	23 ± 12
–	–	TNP KLH	13 ± 9
+	–	"	500 ± 64
+	Spleen	"	33 ± 24
+	ALS	"	586 ± 93
+	ATx	"	520 ± 140
+	ALS + ATx	"	10 ± 6
+	ALS + ATx (αLy-1 + C′)	"	140 ± 110
+	ALS + ATx (αLy-3 + C′)	"	863 ± 140
+	ALS + ATx (αIa^k + C′)	"	13 ± 9
+	ALS + ATx (C′)	"	53 ± 28

Results from a single experiment. Five experiments of this type were performed. Phenotype of suppressor cell precursor is $Ly\text{-}1^-2^+3^+Ia^-$.

TABLE IV
Membrane Phenotype of Suppressor Cell Amplifier

Helper cells	Suppressor cells	Antigen	IgM response (AFC/culture ± SE)
–	–		223 ± 78
–	–	TNP KLH	203 ± 92
+	–	"	923 ± 84
+	Spleen	"	103 ± 58
+	ATx	"	676 ± 94
+	ALS	"	716 ± 153
+	ALS + ATx	"	256 ± 86
+	ALS (αLy-1 + C′) + ATx	"	496 ± 52
+	ALS (αLy-2 + C′) + ATx	"	453 ± 66
+	ALS (αLy-3 + C′) + ATx	"	793 ± 297
+	ALS (αIa^k + C′) + ATx	"	140 ± 74
+	ALS (C′) + ATx	"	162 ± 93

Results from a single experiment. Five such experiments were performed. Phenotype of suppressor cell amplifier is $Ly\text{-}1^+2^+3^+Ia^-$.

treated cells with an equal number of ATx cells before suppressor cell induction and assay (17). In four out of five experiments the phenotype was $Ly\text{-}1^+2^+3^+Ia^-$. In the fifth experiment, the phenotype was $Ly\text{-}1^+2^-Ia^-$. In this experiment anti-Ly-3 was not used and since the anti-Ly-2.1 serum has the lowest titer of all the antisera used, we feel that the $Ly\text{-}1^+2^+3^+Ia^-$ phenotype found in the majority of experiments may be more accurate.

Discussion

It is striking that T-effector cell induction always appears to require T-T interaction. This has been reported for the graft versus host response (20), killer cell response (6, 7), helper cell response (8–10), suppressor cell response (10, 19), and delayed hypersensitivity (21). A necessary step in the verification of postulated interactions is the ability to clearly distinguish the separate cell types involved. This was relatively simple in the case of T-B interactions (reviewed in

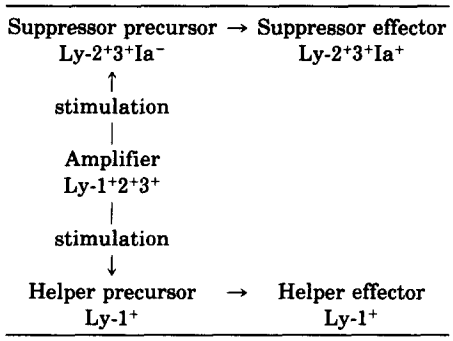

FIG. 1. Scheme of cell interactions and Ly phenotypes for helper and suppressor cell induction.

22), but for T-T, or T-macrophage interactions, markers for T-cell subsets are particularly useful. In this study we used anti-Ly and Ia antisera to establish the identity of cells involved in the generation in vitro of helper or suppressor T cells.

The results obtained show clearly that in this in vitro system, at least two T-cell types are involved in the differentiation of helper or suppressor T cells. The phenotypes are summarized in Fig. 1. In each case the Ly phenotype of the precursor cell, found in ATx spleen, is the same as the effector cell. In the case of helper cells this is Ly-1^+, as has previously been shown by others (2, 3). Suppressor cells have the Ly-2^+3^+ phenotype (5), which has also been found in other systems (23, 24) and can therefore be assigned to the small (less than 10% of peripheral T cells, reference 3) pool of 'T_{cs}' cells (T cytotoxic and suppressor) (25).

More interestingly, we have shown that the amplifier cell belongs to the Ly-$1^+2^+3^+$ pool of 'T_E' (T early) cells. In one experiment out of five the amplifier typed as Ly-1^+2^-, the significance of this result is not clear. In no case was the phenotype Ly-$1^+2^-3^-$, and we attribute the occasional failure of anti-Ly-2.1 to eliminate the response to the low titer of some batches of antiserum and the fact that the assay system requires almost complete elimination of amplifiers before an effect can be seen. (This is because the optimal ratio of ALS/ATx spleen cells ranges from 3:1 to 1:3, and in these experiments only a 1:1 ratio was used). Alternatively, it may be that the amplifiers are in transition from the Ly-$1^+2^+3^+$ to the Ly-1^+ phenotype. It is likely that this type of transition does occur, and a progression from Ly-$1^+2^+3^+$ to Ly-2^+3^+ has been suggested for killer T cells (26). It is intriguing that all the ambiguous experiments were on the phenotype of suppressor cell, not helper cell, amplifiers. If the later phenotype of suppressor amplifiers was Ly-1^+ then the interaction of an Ly-1^+ with an Ly-2^+3^+ precursor would be identical to the interaction of Ly-1^+ and 2^+3^+ cells during killer cell differentiation (4), and it has been suggested that suppression might operate through killing of activated helper cells (26). We do not share this view since we have shown that the Ia surface phenotype of effector suppressor cells differs from that of killer cells (27).

In this report we show that in the majority of experiments the Ia phenotype of the suppressor precursor is Ia^-, while the effectors are consistently Ia^+ (Fig. 1).

Since in one experiment the suppressor precursors were Ia^+, it may be that for this cell lineage the amount of Ia expressed at the membrane increases with the activation or the sensitivity of the cell to lysis changes. The presence of Ia on suppressor cells would be in keeping with the finding of suppressor factors containing Ia determinants (28, 29).

There is controversy as to whether helper cells are also Ia^+. In our hands, under conditions in which suppressor cells are consistently eliminated by anti-Ia^k, helper cells are not (unpublished data); other authors have presented similar findings (30, and footnote 2), but partial elimination of helper activity by anti-Ia sera has also been reported (31). Resolution of these differences will probably result from better characterization of anti-Ia sera and the genetic loci involved.

The data presented here confirm our earlier report of a requirement for T-T interaction for optimal activation of T helpers and suppressors. Although there is an apparent discrepancy between our results showing the humoral response to be sensitive to both anti-Ly-1 and Ly-2 or 3 sera and previous data showing an effect only of anti-Ly-1 (2, 3), a number of possible explanations might account for this. First, the earlier data relates to the response to SRBC (sheep red blood cells), and it may be that there are larger numbers of precursor T_2 cells (primed) for this antigen than for the soluble protein we have used. Secondly, there is evidence to suggest that the interactions of cells involved in responses to particulate antigens differ from those in the response to soluble proteins (32, 33). Even in the case of SRBC, however, it is known that the primary response declines with time after ATx (8), suggesting that T_1 (or T_E) cells may be required when the number of helpers becomes lower.

In another study, the ability to generate helper memory to bovine serum albumin showed only a moderate decline when mice were immunized 4 wk after ATx (9). In our experiments, however, only mice thymectomized at least 16 wk previously were used, so that direct comparison is not possible, although by 16 wk post-thymectomy the ability to generate helper memory to SRBC is very substantially reduced (8).

The mechanism of action of the T_E amplifiers is not known. Although the Ly-$1^+2^+3^+$ cell type comprises approximately 50% of peripheral T cells (3), little is yet known of its function. Because ATx only partially depletes this subpopulation (3) and thoracic duct lymph contains approximately 50% of Ly-$1^+2^+3^+$ cells (unpublished data), it seems likely that some Ly-$1^+2^+3^+$ cells are part of the long-lived recirculating pool. Presumably, the Ly-$1^+2^+3^+$ precursors for altered-self (TNP) killers are part of this fraction. The remaining adult thymectomy sensitive cells contain the amplifier activity detected in this study, but it is not known whether Ly-$1^+2^+3^+$ suppressors, capable of nonspecifically abrogating an in vitro SRBC response (34), also belong in this short-lived fraction. Clearly, however, the Ly-$1^+2^+3^+$ pool is heterogeneous, containing both long-lived precursors and short-lived cells which can exert regulatory effects, but whether our amplifiers are future Ly-1^+ and Ly-2^+3^+ helper and suppressor precursors or alternatively represent a separate regulatory subset of Ly-$1^+2^+3^+$ cells is not yet clear. It is known, however, that differentiation of helper cells requires macro-

[2] Press, J. Quoted in reference 31.

phages and macrophage-derived Ia-antigen factors (33), while suppressor differentiation does not (19). It may be, therefore, that interaction of an amplifier cell with Ia-antigen initiates the helper pathway while interaction with antigen along leads to suppression. Alternatively, more than one T cell within the Ly-$1^+2^+3^+$ pool may be involved. The use of antisera to the recently described Ly-5, 6, and 7 markers (35, 36) may help to resolve these questions.

Summary

The Ly and Ia phenotypes of T lymphocytes involved in the in vitro generation of helper and suppressor cells were identified. The precursors of both cells are found in adult thymectomized spleen. Helper precursors are Ly-$1^+2^-3^-Ia^-$, while suppressor precursors are Ly-$1^-2^+3^+Ia^-$, although the suppressor effector is Ia^+. In both cases a second 'amplifier' cell is required for differentiation of precursors to occur. This cell is found in anti-lymphocyte serum-treated spleen and has the phenotype Ly-$1^+2^+3^+Ia^-$.

We are grateful to Rosemary Duckett and Margaret Dunkley for expert technical assistance and to Dr. U. Hammerling, Sloan-Kettering Institute, New York, for his gift of anti-Ly sera.

Received for publication 29 September 1976.

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