THE SAME HUMAN ALLOREACTIVE T CELL CLONE CAN HELP BOTH B LYMPHOCYTES AND SPECIFIC CYTOTOXIC PRECURSORS

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It is well known that the generation of specific cytolytic activity during Mixed Lymphocyte Reaction (MLR) requires cooperation between cytotoxic T cell precursors (CTLp) and T helper cells (1-2). In humans these two cells can be distinguished and separated using the monoclonal antibody 5/9, which reacts with an antigenic determinant present on the helper-inducer containing subset and absent from CTLp (3).

On the other hand it has been demonstrated that during MLR, help is also generated for the activation of B cells (4-6).

In the present experiments we have used cloned alloreactive T cells to establish whether a single T helper cell can help both B lymphocytes and specific cytotoxic precursors.

Materials and Methods

Cell Suspensions. Peripheral blood mononuclear cells (PBM) were isolated by Ficoll-Hypaque centrifugation from HLA-A, -B, -C, -DR typed donors. PBM were used as stimulator cells after irradiation (3,000 R from a 137Cs source) or were rosetted with neuraminidase-treated sheep erythrocytes and fractionated on Ficoll-Hypaque into non-T cells, containing B cells and monocytes, and T cells (7). The latter were further fractionated using ox erythrocytes coated with the 5/9 monoclonal antibody as described previously (2). The 5/9- T cell fraction was used as a source of CTLp and was itself unable to generate any cytotoxicity when cultured with allogeneic stimulator cells. The 5/9+ fraction was used as a source of T helper cells.

Culture Media. The medium used was RPMI-1640 supplemented with 2 mM L-glutamine, 1% non-essential aminoacids, 1% pyruvate, 50 μg/ml gentamycin, 5 × 10^-5 M 2-mercaptoethanol, and 10% fetal calf serum (FCS). As a source of T cell growth factor (TCGF) a supernatant of PHA-activated tonsil cells was used as previously described (8).

T Cell Clones. Alloreactive clones were isolated from a secondary MLR (donor M anti-A) by cloning the T blasts at 0.3 cells/well in the presence of stimulator cells and 10% PHA-superpant as described elsewhere (6, 8). After 2 wk the growing wells, obtained with an efficiency of 60%, were further expanded with TCGF and screened for their capacity to proliferate in response to the original stimulator in the absence of exogenous growth factors. The clones MA-1 and MA-8 used in the present study have a different allospecificity as revealed by family studies and have been recloned several times during

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a period of one year. They are OKT3*, 4*, 8- (9) and 5/9*. The clones can be maintained in culture by supplementing the medium with TCGF in the absence of stimulator cells, but periodical restimulations with the original alloantigen are required in order to maintain cell growth for long periods.

**Helper Assay for the Generation of Specific CTL.** Cultures set up in 1.5 ml RPMI-FCS in flat-bottom 24-well plates containing 5 x 10^5 irradiated PBM as stimulator cells, 7 x 10^5 5/9- T cells as CTLp and, as a source of Th cells, either 5/9* T cells (2.5 x 10^5) or specific T cell clones (5 x 10^4-5 x 10^5 nonirradiated or 10^5 irradiated). After 7 d the cultures were harvested and specific cytotoxicity was tested against PHA-TCGF blasts derived from the stimulator or from unrelated donors that did not share any HLA specificity with the stimulator. Briefly, varying numbers of effectors (9.4 x 10^3-3 x 10^4) were incubated in V-bottom microplates with 5 x 10^5 51Cr-labeled PHA-TCGF blasts in a final volume of 200 µl, thus ensuring effector/target ratios ranging from 1.8:1 to 60:1. The plates were centrifuged at 100 g for 5 min and incubated for 4 h at 37°C. The plates were centrifuged again and 100 µl of the supernatant was collected and counted for 1 min in a γ counter. Specific lysis was calculated according to Cerottini et al. (10). Lytic Units (LU) were calculated according to Brunner et al. (11) by plotting the percentage of specific 51Cr release vs. the number of effector cells. The number of effector cells required for 30% lysis (estimated by the linear regression analysis) was defined as 1 LU.

**Helper Assay for B Cell Activation.** Cultures were set up in 150 µl RPMI-FCS in U-bottom 96-well microplates containing 2 x 10^4 non-T cells and various numbers of helper T cells (allogeneic 5/9+ fraction or specific alloreactive T cells clones). In some experiments allogeneic 5/9- T cells were also added in culture. After 10 d total IgG, IgA, and IgM were measured in the culture supernatant with a class-specific enzyme immunoassay as previously described (8).

**Results**

Alloreactive clones with helper function on B cell activation were isolated from a secondary MLR (donor M anti-A) as previously described (6). In the present experiments, two of such clones have been tested as a source of helper cell for the generation of specific cytolytic activity and for the activation of B cells.

Peripheral blood T cells from the same donor from which the alloreactive clones were derived were separated in the 5/9+ fraction (containing the helper cells) and the 5/9- fraction (containing CTLp). As previously shown (2), neither of these subpopulations alone was able to generate cytotoxic activity when cultured with allogeneic cells, while the reconstituted population showed all the cytolytic activity of the unseparated T cells (Table I). We were therefore able to test the capacity of alloreactive T cell clones to substitute for autologous 5/9+ T cells in the generation of help for a cytotoxic response against the stimulator cells. As shown in Table I, very low numbers of T cells from the two clones tested provided the same, and even better, help for CTL generation than autologous 5/9+ T cells. Appropriate controls showed that the clones themselves were not cytotoxic.

In the same experiments the alloreactive clones or the 5/9+, 5/9- T cell fractions were cultured with B cells carrying the relevant alloantigens. Total Ig production was measured in the 10-d culture supernatant. The results reported in Table II show that the same clones that help CTLp also induced a strong polyclonal B cell activation, with production of very high levels of IgM, IgG, and IgA. Interestingly some help was provided also by the 5/9+ T cells. On the other hand the addition of the 5/9- subset always inhibited the B cell activation, the extent of this inhibition paralleling the development of the cytotoxic response.
### Table I

**Alloreactive Helper Clones Can Substitute for Autologous 5/9+ T Cells in the Generation of a Specific Cytotoxic Response**

| Culture conditions | CTLp (donor M) | Stimulator (donor A) | Cytolytic activity on target A (LU/well) |
|-------------------|----------------|---------------------|----------------------------------------|
| Helper cells (donor M) | 5/9+ | | |
| | 2.5 X 10^4 5/9+ | + | 0 |
| | 5 X 10^4 MA-1 | + | 14 |
| | 5 X 10^4 MA-1 (Rx)* | + | 18.4 |
| | 2 X 10^4 MA-8 | + | 24.6 |
| | 10^5 MA-8 | + | 23.1 |
| | 10^5 MA-8 | + | 31.5 |

Helper T cells (5/9+ fraction from peripheral blood or T cell clones MA-1 and MA-8 specific for alloantigens of donor A) were cultured in the presence or absence of 7 x 10^5 autologous 5/9- T cells (as a source of CTLp) with 5 x 10^5 irradiated PBM from donor A in 1.5 ml RPMI-FCS. After 7 d specific cytolytic activity against PHA-TCGF blasts from donor A was determined.

* In some experiments the T cell clones were γ-irradiated (3,000 R).

### Table II

**Alloreactive Helper Clones and 5/9+ T Cells Induce a Polyclonal B Cell Activation That Is Inhibited by 5/9- T Cells**

| Culture conditions | Ig produced |
|-------------------|-------------|
| Helper cells (donor M) | 5/9- T cells (donor M) | Non-T cells (donor A) | IgM | IgG | IgA |
| | 2 X 10^4 5/9+ | + | + | 0.2 | 0.3 | <0.1 |
| | 5 X 10^4 MA-1 | + | + | 6.5 | 2.1 | 0.6 |
| | 10^4 MA-1 (Rx)* | + | + | 1.1 | 1.5 | 0.2 |
| | 10^4 MA-1 (Rx)* | + | + | 125 | 90 | 42 |
| | 10^4 MA-8 | + | + | 120 | 85 | 30 |
| | 10^5 MA-8 | + | + | 6 | 5.5 | 2 |
| | 10^5 MA-8 | + | + | 130 | 10 | ND |
| | 10^5 MA-8 | + | + | 8.9 | 1 | ND |

Helper T cells were cultured in the presence or absence of 5 x 10^4 autologous 5/9+ T cells with 2 x 10^5 allogeneic non-T cells as a source of responding B cells in 150 μl RPMI-FCS. Total Ig were measured in the 10-d culture supernatant. Non-T cells alone produced <0.5 μg/ml total Ig.

* γ-irradiated.

As a control, the addition of the 5/9+ fraction never did result in an inhibition of the B cell response (data not shown).

The above findings indicate that a single T cell clone can completely substitute for autologous 5/9+ T cells in the generation of help for both CTLp and B cells. It should be noted, however, that a titration of the number of cloned T cells in both culture systems was required in order to demonstrate both effects. In fact, the optimal cell concentration is much lower for the cytotoxicity assay than for the B cell assay and also differs from one clone to the other. It is possible that an overgrowth of the helper clone may interfere with the generation of specific cytotoxic T cells, for instance by competing for TCGF (12). As the helper activity of the cell clones was found to be radioresistant (Table II), in the following
experiments we used $10^6$ 3,000-R-irradiated cloned T cells for the cytotoxic helper assay with similar and reproducible results.

It has been previously demonstrated that once activated by the relevant alloantigen, the helper clones are able to provide unrestricted help to B cells (6, 13). Therefore in the next series of experiments we investigated whether the alloreactive clones could provide help only to autologous, or also to unrelated CTLp. As evident from a representative experiment reported in Fig. 1, when the clones were cultured in the presence of the appropriate stimulator, the same extent of help was provided to autologous and unrelated 5/9- cells for a cytotoxic response against the stimulator. It is remarkable that also in this case the cytotoxic response obtained using clones as the only source of T helper cells is higher than that obtained using the autologous 5/9+ fraction.

The specificity of the CTL response generated was investigated by testing for cytotoxicity against PHA blasts from the stimulator (donor A) and from unrelated donors that did not share any HLA antigen with A. Fig. 2 shows that the CTL

![Figure 1](image1.png)

**FIGURE 1.** Alloreactive clones in the presence of the appropriate stimulator can help both autologous and unrelated CTLp. 5/9- T cells were cultured with stimulator cells from donor A alone (○) or in the presence of γ-irradiated T cells from clone MA-1 (●), MA-8 (△) or with their 5/9+ fraction (■). a, 5/9- (and 5/9+) T cells were from donor M autologous to the helper clones or b and c, from two donors unrelated to A or M. Specific cytotoxicity against PHA-TCGF blasts of donor A was measured on day 7.

![Figure 2](image2.png)

**FIGURE 2.** The cytolytic activity elicited by the helper clones is specific for the stimulator cells. (a) Clone MA-8 was cultured with the specific stimulator (A) and 5/9- T cells (donor P). After 7 d the cultures were tested for specific cytolytic activity against the stimulator A (●) and against an unrelated donor G (△) and S (■). (b) Same culture conditions except that irradiated PBM of donor G and S were also added at the initiation of cultures.
generated were able to lyse only the stimulator cells. However, when another stimulator unrelated to A was also added at the initiation of cultures, specific CTL were generated also against these cells.

Discussion

The present experiments demonstrate that a single T helper cell, following recognition of the specific alloantigen, can help both B lymphocytes and specific cytotoxic precursors in a way that is neither antigen-specific nor MHC-restricted. These data are in apparent contrast with a recent report showing that alloreactive T cell lines may either help B cells or amplify cytotoxic responses induced by suboptimal doses of stimulator cells (14). On the other hand, it has been reported that a single T cell clone reactive against a lymphoblastoid cell can produce soluble factors that induce the terminal differentiation of the same B cell line and help the generation of cytotoxic T cells (15). In the present experiments, the availability of T cell clones and of the 5/9- fraction allowed to approach the problem in unambiguous terms and to formally demonstrate that the same T cell clone can serve as the only source of T helper cells for the induction (and not only the amplification) of both cytotoxic and antibody responses. It should be also noted that, in order to demonstrate the help of a T cell clone for both B cells and CTLp, it was necessary to carefully titrate the helper cells. This may be an important methodological point as far as the functional properties of T cell clones are concerned. It is in fact possible that an apparent heterogeneity at the clonal level simply reflects the degree of expression in a given clone of two functional properties that are normally and independently distributed in the T cell population.

The above experiments also demonstrate that the polyclonal B cell activation induced by the alloreactive helper clones (or by the 5/9+ helper fraction) is inhibited by the addition of 5/9- T cells. As in the same cultures specific cytotoxicity against the responder B cells was generated, it is likely that this inhibition is mediated by cytotoxic T cells. These data strengthen the necessity of cloning the T helper cells (or isolating the helper fraction with monoclonal antibodies) in order to demonstrate a positive allogeneic effect on Ig production.

Finally, the possibility of substituting for the T helper cells with an alloreactive clone allows the stimulation of any CTLp against any target and is suitable for the analysis of the cytotoxic potential under conditions of non-limiting help. The latter point may be relevant for the study of cases such as patients receiving allogeneic grafts or immunosuppressive therapy that are unable, apparently because of a lack of T helper cells, to generate a cytotoxic response.

Summary

Human alloreactive proliferating T cell clones have been compared for their capacity to provide help for B cell activation and the generation of a specific cytotoxic response. The results demonstrate that, when triggered by the relevant alloantigen, the same T cell clone can induce a strong polyclonal B cell activation and serve as the only source of helper cells for the generation of a specific cytotoxic response by any source of CTL precursors against any stimulator cell present in culture.
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