

DELINEATION OF AN IMMUNOREGULATORY AMPLIFIER  
POPULATION RECOGNIZING AUTOLOGOUS Ia  
MOLECULES

Analysis with Human T Cell Clones

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Human T cell clones and monoclonal antibodies directed at their surface glycoproteins have provided a considerable amount of information about the nature of recognition structures on alloreactive, viral-specific and soluble antigen-specific human T lymphocytes (1-6). In each case, the antigen receptor of these cells has been shown to be a T3-Ti molecular complex that consists of one clonally unique 90-kD Ti molecule in noncovalent association with the monomeric 20/25-kD T3 molecule (7-10). While autoreactive T cells have been defined in both human and murine systems as well, their recognition elements, in contrast to cells with the above specificities, have not been identified (11-18). However, target molecules from these latter cells could be either the antigen receptors on other effector populations or alternatively, autologous surface components including MHC gene products.

To investigate autoreactive cells at the clonal level, we utilized a major histocompatibility (MHC)<sup>1</sup> restricted/antigen-specific inducer clone, termed RW17C in the present study, as a stimulus for proliferation, cloned the responding T cells and then studied their specificity and function. The results, to be reported below, indicate that the majority of such clones are derived from the T4+ subset and are reactive with autologous MHC class II molecules. These "autoreactive" cells provide inducer as well as amplifier function for B cell Ig production in the absence of exogenous antigen since only self-MHC recognition is required for activation. Functional and biochemical analysis of such clones demonstrates that, similar to conventional "antigen-specific" clones, they use a

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<sup>1</sup> *Abbreviations used in this paper:* AC, autoreactive clones; AMLR, autologous mixed lymphocyte reaction; E, erythrocytes; EBV, Epstein-Barr virus; FITC, fluorescein isothiocyanate; IL-2, interleukin 2; MHC, major histocompatibility complex; NHS, normal human serum; NK, natural killer cells; PBMC, peripheral blood mononuclear cells; RIPA, radioimmune precipitation assay; RWAGE, ragweed antigen E; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

comparable T cell receptor comprised of a T3-Ti molecular complex for recognition of autologous MHC.

### Materials and Methods

*Isolation of Lymphoid Populations.* Macrophages were obtained from mononuclear populations by standard adherence techniques. Subsequently, macrophage-depleted mononuclear cells were separated into erythrocyte (E) rosette positive (E+) and E rosette negative (E-) populations with 5% sheep E (Microbiological Associates, Bethesda, MD). The rosetted mixture was layered over Ficoll-Hypaque and the recovered E+ pellet was treated with 0.155 M NH<sub>4</sub>Cl to lyse erythrocytes. The T cell population obtained was >95% E+ and >90% reactive with monoclonal antibody anti-T3, which defines an antigen present on all mature peripheral T lymphocytes (19). The E- population was further fractionated into B cell populations by complement-mediated lysis with anti-T3 and anti-Mo1 (20). Reanalysis of antibody and complement lysis of E- cells demonstrated that the B cell population was >94% reactive with anti-B1 (21).

*Generation and Culture of Human T Cell Clones.* The human T cell clone RW17C was derived by stimulating peripheral blood lymphocytes of a healthy donor with ragweed antigen E (RWAGE) (Worthington Diagnostic Systems, Freehold, NJ) followed by cloning as previously described (22). Briefly, peripheral blood mononuclear cells (PBMC) were incubated with RWAGE (Worthington Diagnostics) for 6 d and then cloned in a semi-solid agar system. Individual colonies were expanded in liquid culture by restimulation with RWAGE, 5,000 rad irradiated autologous PBMC, and interleukin 2 (IL-2)-containing supernatants for >8 mo. Further cloning was performed by limiting dilution technique at one cell/well. The surface phenotype of RW17C was T1+T3+T4+T8-T11+T12+Ia+, as analyzed by indirect immunofluorescence on an Epics V cell sorter (Coulter Electronics, Hialeah, FL).

The autoreactive human T cell clones (AC) were derived by stimulating lymphocytes of the same donor as RW17C with autologous T cell clone RW17C and cloning and recloning responding cells (22). To this end, PMBC were stimulated with an equal concentration of irradiated RW17C for 5 d in final culture medium RPMI 1640 with 10% human AB serum. Cloning of activated cells was performed in a two-layer soft agar system. After 6-9 d, agar colonies were individually transferred into round-bottom microtiter plates (Costar, Cambridge, MA) containing 0.1 ml of irradiated feeder cell suspension ( $0.75 \times 10^6$  autologous whole mononuclear cells and  $0.5 \times 10^6$  RW17C per ml). At this time, the culture medium was supplemented with 5% IL-2. Subsequently, the cultures were expanded by feeding every 2-3 d with IL-2-conditioned medium and weekly restimulation with irradiated feeder cells. Recloning was performed by limiting dilution in terasaki plates at 0.3 cells per well in the presence of 5% IL-2 and irradiated feeder cells. Several subclones were expanded and maintained in culture for 6 months. The surface phenotype of individual AC clones was either T1+T3+T4+T8-T11+T12+TQ1-Ia+ or T1+T3+T4-T8+T11+T12+TQ1-Ia+.

*IL-2.* IL-2-containing supernatants were produced by stimulating whole peripheral mononuclear cells with phytohemagglutinin and phorbol myristate acetate in the presence of inactivated B cell line Laz 156, as described (17).

*Generation of Monoclonal Antibodies and Heteroantisera.* The following monoclonal antibodies were utilized in the present study and purified as previously described (6). Anti-T1 (reactive with the total T cell population); anti-T3 (all mature T lymphocytes and a fraction of thymocytes); anti-T4 (human inducer population); anti-T8 (human suppressor population); anti-T11 (E+ human lymphocyte); anti-T12 (all mature T cells); anti-TQ1 (reactive with 70-85% of T4+ inducer cells) (18). Anti-p29,34 is a turkey heteroantiserum against human Ia antigen (monomorphic region of MHC class II gene product) and was provided by E. Yunis (Dana-Farber Cancer Institute) and prepared as described (24). w6/32 anti-HLA monoclonal antibody was purchased from Sera laboratories (Sussex, England) (20). Anti-Ia framework (monomorphic, 9-49) was kindly provided by Dr. Robert Todd (Dana-Farber Cancer Institute).

*Phenotypic Analysis of T Cell Surface Antigens.* Phenotypic analysis of T lymphocytes was performed by means of indirect immunofluorescence with a panel of monoclonal antibodies and goat anti-mouse F(ab')<sub>2</sub> fluorescein isothiocyanate (FITC) on an Epics V cell sorter (Coulter Electronics). For quantification by flow cytometry, the cells were incubated with saturating concentrations of monoclonal antibody and goat anti-mouse FITC. Quantification by this method is possible since fluorescence measured by a fluorescence-activated cell sorter is proportional to the number of bound fluorescein molecules per cell and is linear with the channel number using a linear amplifier (26). The present quantitations are based on a valid calibration curve linear and logarithmic fluorescence on the Epics V cell sorter (27). Since homogeneous fluorescence peaks were essentially gaussian on the log scale, their average fluorescence intensities were determined as the geometric mean (that is, the arithmetic mean channel number of log amplified intensities). Each cell population was analyzed several times with consistent results (variations <10%).

*Modulation of Surface Antigens by Monoclonal Anti-T3 Antibodies.* AC3 cells at  $1 \times 10^6$ /ml were incubated with saturating amounts of anti-T3 antibody for 18 h at 37°C in final culture medium RPMI 1640 plus 10% human AB serum. Subsequently, the cells were washed three times and analyzed for expression of T3 by means of indirect immunofluorescence on the FACS before assessment of their functional properties.

*Radiolabeling and Immunoprecipitation of T Cell Surface Antigens.* Cell surface iodination of T cell clones was performed with Na <sup>125</sup>I (New England Nuclear, Boston, MA) and catalyzed by lactoperoxidase. To  $20 \times 10^6$  cells suspended in 1 ml PBS were added successively 10  $\mu$ l glucose (0.5 mol/liter), 5  $\mu$ l NaI ( $5 \times 10^4$  mol/liter), 10  $\mu$ l lactoperoxidase (2 mg/ml), 1 mCi Na <sup>125</sup>I, and 20  $\mu$ l glucose oxidase (7.5 mU/ml). This mixture was incubated for 15 min at room temperature followed by addition of 100  $\mu$ l NaI (1 mol/l). After an additional 2 min of incubation, cells were washed four times in Hank's balanced salt solution. The final pellet was lysed in 500  $\mu$ l 1:5 diluted radioimmune precipitation assay (RIPA) stock solution containing 1% Triton X-100 (RIPA stock solution: 0.1 mol/l NaH<sub>2</sub>PO<sub>4</sub>, 1 mmol/l phenylmethylsulfonyl fluoride, 10 mmol/l EDTA, 10 mmol/l EGTA, 10 mmol NaF, 1% deoxycholate sodium salt, 200 KIU/ml aprotinin, pH 7.2). The suspension was centrifuged for 5 min at 1,120 g and the resulting supernatant was precleared twice using monoclonal antibody anti-T6 covalently linked to CnBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), each for 60 min at 4°C and subsequently centrifuged 5 min at 1,120 g. Precleared lysates were incubated with monoclonal antibodies bound to CnBr-activated Sepharose 4B for 2 h at 4°C. The resulting precipitate was subsequently washed five times in RIPA solution (stock 1:10 diluted) plus 1% Triton X-100 resuspended in gel buffer (0.125 mol/liter Tris-HCl, pH 6.8, containing 10% glycerol, 3% sodium dodecyl sulfate [SDS] in the presence or absence of 5% 2-mercaptoethanol) and boiled for 5 min. SDS polyacrylamide gel electrophoresis (PAGE) was performed on a continuous vertical slab gel (10% polyacrylamide) for 14 h according to a modification of the Laemmli procedure (28).

*Proliferative Studies.* The T cell clones and irradiated stimulator cells were plated into round-bottomed microtiter plates in a total volume of 200  $\mu$ l of supplemented RPMI 1640 containing 10% human AB serum when IL-2-conditioned medium was used (final concentration, 5–10%). After a 24-h or 72-h incubation at 37°C, the various cultures were pulsed with 1  $\mu$ Ci of tritiated thymidine (<sup>3</sup>H-TdR) (Schwarz/Mann, Div. of Becton Dickinson, Orangeburg, NY) and harvested 18 h later on a MASH II apparatus (M.S. Bioproducts, Walkersville, MD). <sup>3</sup>H-TdR incorporation was then measured in a scintillation counter (Packard Instruments, Downers Grove, IL). Each value represents the mean of triplicates  $\pm$  standard deviation (SD).

*In Vitro Analysis of Regulatory Functions on IgG Production.* AC3 or RW17C cells ( $30 \times 10^3$ ) were cultured with B lymphocytes ( $5 \times 10^4$ ) with or without RWAGE (final concentrations 5  $\mu$ g/ml) in a total volume of 0.20 ml in 96-well U-shaped tissue culture plates (Flow Laboratories, McLean, VA) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% FCS (M.A. Bioproducts). After 7 d incubation at 37°C in a humidified atmosphere containing 6% CO<sub>2</sub>, the wells were harvested by pooling the cells and supernatants for replicate wells and storing at -20°C before assay for IgG synthesis. The

amounts of IgG secreted varied considerably among individual experiments (e.g. maximal IgG concentration between 1 and 15  $\mu\text{g}/\text{ml}$ ). Nevertheless, in all experiments RW17C or AC3, respectively, induced a 25–30-fold IgG secretion as compared with the autologous B cell and RWAGE.

*RIA for IgG.* A solid phase RIA for IgG was performed in disposable flexible polyvinyl microtitration plates containing 96 U-bottomed wells (Cat. No. 1-220-24B, Dynatech, Alexandria, VA). Specifically, individual wells of the plates were coated with 100  $\mu\text{l}$  of a 1:20 dilution of the immunoglobulin fraction of rabbit anti-human IgG (chain specific) antiserum (Dakopatts A/S, Denmark) overnight at room temperature. The coating antibodies were aspirated and saved for re-use. The plates were washed three times with diluent of PBS containing 10% BSA for 1 h at room temperature. The plates were washed once and at this stage could be stored at 4°C in PBS 1% BSA containing sodium azide.

Multiple point standard curves were prepared using doubling dilutions of normal human serum (NHS). The IgG content of NHS was standardized against the Australian standard reference serum for immunoglobulin (ASPS 78/1). Aliquots (100  $\mu\text{l}$ ) of standard or culture fluids were added to individual wells in triplicate and allowed to stand overnight at room temperature. The plates were then washed three times with diluent and 100  $\mu\text{l}$  containing 5 ng of  $^{125}\text{I}$ -labeled IgG solid phase adsorbed rabbit anti-human IgG antiserum (Calbiochem-Behring Corp., La Jolla, CA) was added to each well. The antibodies were labeled with sodium iodide ( $^{125}\text{I}$ ) for protein iodination (Amersham International, Amersham, UK) using Enzymobead Reagent (Bio-Rad Laboratories, Richmond, CA) to a specific activity of 4,000 cpm/ng. After 4–6 h incubation, the plates were washed 10 times with PBS. The individual wells were cut apart and counted in an auto-gamma scintillation spectrometer (Packard). Standard curves in this RIA were linear between 1 and 250 ng IgG per ml. Whenever the concentration of IgG in a culture supernatant exceeded 250 ng/ml, it was diluted so that it would fall in this range.

## Results

*Generation of Autoreactive Clones.* Evidence exists to support the notion that autoreactive T cells circulate in human peripheral blood (11–15). However, neither the precise nature of the recognition structures on the responding population nor the target antigens that serve as their stimulus have been defined. To address these questions at the clonal level, a series of T cell clones were generated against an autologous, antigen-specific T4+ clone, termed RW17C. For this purpose, peripheral blood mononuclear cells (PBMC) were sensitized in vitro to irradiated RW17C for 6 d and cloned in a two-layer soft agar system. The agar colonies were picked, expanded, and then recloned by limiting dilution as previously described. After 1 month of in vitro expansion, cultures were screened for reactivity with anti-T4 and anti-T8 monoclonal antibodies by indirect immunofluorescence on an Epics V cell sorter. Of the initial cultures screened, 20 AC showed a homogeneous phenotype in terms of anti-T4 and anti-T8 reactivity. 13 of these expressed the T4 surface determinant, whereas 7 expressed the T8 antigen. The former (phenotype: T3+T4+T8–T11+Ia+) were maintained in in vitro continuous culture for >6 months and are the subject of the present report.

*Proliferative Responses of Autoreactive Clones.* Because the resulting clones might, in principle, be directed either at surface structures unique for RW17C (i.e. RW17C T cell receptor) or autologous lymphoid structures present on other T and/or B cells from the same individual, the ability of individual autoreactive clones to proliferate to RW17C and an autologous Epstein-Barr virus (EBV) lymphoblastoid line termed Laz 509 were tested. The results of proliferation

studies for seven representative clones is shown in Table I. Although our initial expectation was that AC1-7 might proliferate to RW17C, they responded minimally despite the fact that the AC clones were stimulated in the bulk cultures with RW17C. In contrast, all seven clones proliferated to the autologous EBV-transformed lymphoblastoid line Laz 509. Moreover, the specificity of these anti-clones for autologous cells was clear because they did not proliferate to allogeneic B lymphoblastoid lines (Laz 156). It is possible that IL-2 consumption by RW17C accounted for its apparent inability to stimulate AC1-13; however, exogenous IL-2 activity could be detected in culture supernatants of irradiated RW17C cells for 48 h (data not shown).

Since it was previously shown that the majority of cytotoxic T4+ clones were directed at unique determinants on class II molecules (1-3), we examined the ability of monoclonal antibodies directed against class I and class II MHC gene products to block proliferation of the autoreactive clones. As shown in Fig. 1, preincubation of the irradiated Laz 509 stimulator cells with an anti-Ia monoclonal antibody to an invariant region of the 29/34 structure (or anti-p29, 34 heteroantiserum) diminished the proliferation of a representative clone by ~50% (40,000 cpm vs. 90,000 cpm; lane 5 vs. 4). In contrast, the monoclonal anti-HLA antibody had no effect (lane 6). Moreover, the inhibition of proliferation mediated by anti-Ia antibody was specific for the antigen-induced proliferative response of AC3 because IL-2-mediated proliferation was not significantly diminished (52,000 vs. 46,000 cpm; lanes 7 and 8). Similar findings were observed with all the other AC clones tested. That these AC clones proliferated to autologous B lymphocytes in xenogeneic serum as well as autologous or allogeneic human serum, makes it unlikely that any serum component was being recognized in conjunction with self-class II molecules. Furthermore, since non-T cells from several HLA-D region matched allogeneic individuals could not trigger the AC3 clone to proliferate, it is probable that the epitope of the class II molecule being recognized is of a private specificity and more restricted than that defined by serologic means (data not shown).

Next the capacity of different autologous cell populations to trigger the AC

TABLE I  
*Reactivity of AC Clones Against an Autologous EBV-Transformed Lymphoblastoid B Cell Line*

	Medium	Specific autologous T cell clone (RW17C)	Autologous EBV-transformed lymphoblastoid B cell line (Laz 509)	Allogeneic EBV-transformed lymphoblastoid B cell line (Laz 156)
AC1	60 ± 22	123 ± 106*	3,679 ± 256	213 ± 51
AC2	62 ± 8	125 ± 98	3,166 ± 118	65 ± 10
AC3	71 ± 33	50 ± 20	3,182 ± 120	189 ± 68
AC4	101 ± 46	134 ± 112	2,554 ± 102	134 ± 50
AC5	47 ± 21	170 ± 78	3,858 ± 288	231 ± 10
AC6	85 ± 30	56 ± 21	1,597 ± 108	87 ± 17
AC7	45 ± 4	176 ± 78	2,460 ± 93	62 ± 2

\* <sup>3</sup>H-TdR mean ± SD of triplicate samples.

10<sup>5</sup> cells of each AC were cocultured with 15 × 10<sup>5</sup> irradiated cells and subsequently <sup>3</sup>H-TdR incorporation was measured on day 2. Results are expressed as cpm ± SD.

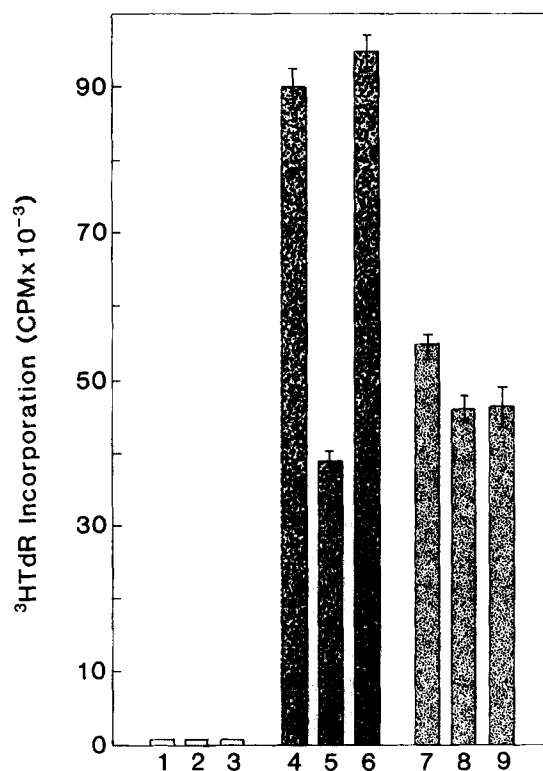


FIGURE 1. Influence of anti-Ia and anti-HLA antibodies on proliferative responses of clone AC3.  $3 \times 10^4$  cells of clone AC3 were cultured with either: 1, medium; 2, anti-Ia; 3, anti-HLA; 4, irradiated Laz 509; 5, irradiated Laz 509 and anti-Ia; 6, irradiated Laz 509 and anti-HLA; 7, 10% IL-2 containing supernatants; 8, 10% IL-2 containing supernatants and anti-Ia; and 9, 10% IL-2 containing supernatants and anti-HLA. Final dilutions of the monoclonal antibodies were anti-Ia, 1:250 and anti-HLA, 1:50. <sup>3</sup>H-TdR incorporation was measured on day 2. Results are expressed as cpm + SD and representative of four such experiments.

clones was determined and compared with their ability to activate the MHC/antigen-specific clone RW17C used in the original stimulation. In addition, given the apparent specificity of the T4+ AC1-13 clones for autologous Ia molecules, binding sites for anti-Ia were quantitated on each stimulator cell by indirect immunofluorescence. As shown in Table II, the representative autoclone AC3 proliferates to autologous B cells, macrophages, and the EBV-transformed B lymphoblastoid line. Moreover, whereas the response to resting T cells, B cells, macrophages, and autologous B lymphoblastoid cells correlated with the number of Ia molecules which they express, little response was induced by activated T cells including RW17C and four other T cell clones of differing specificities which were either T4+Ia+ or T8+Ia+, despite the fact that they expressed 20,000–40,000 Ia sites per cell (Table II, data not shown). Whether the inability of these T cells to induce significant AC3 proliferation was a consequence of their low level of Ia expression or alternatively, secondary to absence of a critical Ia molecule that is required for secondary T cell stimulation is not known at the present time. Similar findings were observed with AC1, 2, 5, and 6. In no cases

TABLE II  
*Clone AC3 Is Activated by Autologous Cells*

Stimulus	Number of Ia molecules*	Proliferation	
		AC3	RW17C
Autologous T cells	≤3,000	90 ± 1	350 ± 172
Autologous T cell clone	40,000	820 ± 6	360 ± 152
Autologous B cells	60,000	4,560 ± 600	113 ± 100
Autologous B cells + RWAGE		4,832 ± 720	1,019 ± 204
Autologous macrophages	120,000	18,882 ± 1,012	430 ± 95
Autologous macrophages + RWAGE		18,954 ± 320	9,754 ± 1,009
Autologous EBV transformed B cell line (Laz 509)	>500,000	82,621 ± 2,532	350 ± 172
Medium	—	78 ± 4	270 ± 150
IL-2	—	54,510 ± 568	3,256 ± 269

\* Binding sites/stimulator cell.

$3 \times 10^4$  cells of AC3 or RW17C were cocultured with  $5 \times 10^4$  irradiated stimulator cells. Ragweed antigen E (RWAGE) was utilized at a final concentration of 20  $\mu$ g/ml and IL-2 at 10% vol/vol. Proliferation was assessed by measuring incorporation of  $^3$ H-TdR on day 2. Number of Ia molecules was calculated as described in Materials and Methods. Results are expressed as cpm  $\pm$  SD.

however, were the autoclones cytotoxic for T cells, B cells, or macrophages (data not shown).

Table II also shows that the representative AC3 autoclone was clearly distinct from RW17C in that AC3 was triggered to proliferate by autologous MHC class II expressing B cells or macrophages alone, whereas RW17C required both autologous B cells and/or macrophages and the exogenous soluble antigen (RWAGE). Note that the autologous macrophages presented RWAGE antigen better than autologous B cells to RW17C as judged by  $^3$ H-TdR (9,700 vs. 1,000 cpm, respectively). Whether this difference relates to the greater expression of autologous class II molecules on the former is not known, but appears likely. Table II further demonstrates that the relative magnitude of the proliferative response to a given stimuli was greater for AC3 than RW17C in all cases.

*Immunoregulatory Function of Autoreactive Clones.* The finding that AC3 and the other AC clones failed to proliferate to RW17C but rather proliferated to B cells and macrophages, suggested that they might, in turn, affect other B cell functions. To test the ability of AC3 to influence IgG synthesis by autologous B lymphocytes, this clone and the MHC-restricted antigen-specific inducer clone RW17C was examined in parallel. To this end,  $5 \times 10^4$  autologous or allogeneic B lymphocytes were incubated for 7 d at 37°C with  $3 \times 10^4$  AC3 or RW17C cells in the presence or absence of ragweed antigen. Subsequently, culture supernatants were harvested and analyzed for IgG in a solid phase radioimmunoassay as previously reported. As shown in Fig. 2, panel A, AC3 induced IgG secretion from autologous B cells (lanes a and b, >14,000 ng/ml) in the presence or absence of exogenous RWAGE antigen. In contrast, allogeneic B cells or autologous or allogeneic macrophages were incapable of inducing Ig secretion in the presence of AC3 (c-f, <500 ng/ml). In addition, lane g indicates that AC3 could induce allogeneic B lymphocytes to produce Ig in the presence of autologous macrophages. In that the latter do not, by themselves, produce Ig when

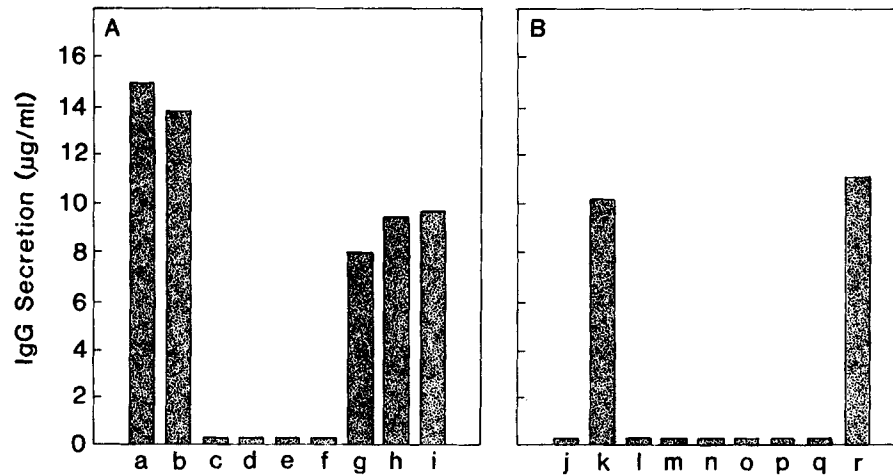


FIGURE 2. Induction of IgG secretion from autologous B lymphocytes by clone AC3 and clone RW17C.  $3 \times 10^4$  cloned T cells (panel A = AC3; panel B = RW17C) were cocultured in 10% FCS with the various stimuli listed below. After 7 d of culture, the wells were harvested and IgG secretion measured by solid phase radioimmunoassay. Results of one of five representative experiments are shown and expressed as  $\mu\text{g/ml}$  of human IgG. SD for triplicate samples was always  $\leq 5\%$ . Stimuli were as follows: (a,j) autologous B cells; (b,k) autologous B cells and RWAGE ( $5 \mu\text{g/ml}$ ); (c,l) allogeneic B cells; (d,m) allogeneic B cells and RWAGE ( $5 \mu\text{g/ml}$ ); (e,n) autologous macrophages; (f,o) allogeneic macrophages; (g,p) allogeneic B cells and autologous macrophages; (h,q) autologous B cells and allogeneic macrophages; (i,r) allogeneic B cells, autologous macrophages and RWAGE.

stimulated by AC3 (lane *e*), these results suggest that the autologous macrophages served to trigger the autoreactive clone that subsequently provides inducer function to allogeneic B cells in a non-MHC restricted fashion. Note that the removal of all peripheral blood T cells from the non-T population with anti-T3 and complement (see Materials and Methods) rules out the possibility that IL-2 secretion by AC3 induced a normal "contaminating" T cell population to produce BCDF and facilitate B cell differentiation.

In contrast to AC3, RW17C provided inducer function for B lymphocytes only in the presence of the soluble antigen RWAGE. Thus, as shown in panel B, RW17C plus autologous B lymphocytes yielded no Ig production (*j*), whereas the same combination plus RWAGE antigen provided  $>10,000 \text{ ng/ml}$  (*k*). In contrast, allogeneic B lymphocytes with or without RWAGE antigen were not induced to secrete Ig (*l* and *m*). Note that as with AC3, allogeneic B lymphocytes could be induced by RW17C to produce Ig but in the latter case this required autologous macrophages and RWAGE (*r*).

Because the specificities of RW17C and AC3 were distinct, it was of interest to determine whether the inductive effects of these two clones were also different. To investigate this question and explore the possibility of a synergistic effect of these two inducer populations, an increasing number of RW17C or AC3 cells were added to autologous B cells individually or together and subsequently, total Ig measured after 7 d. As shown in Fig. 3, Ig production by the combination of B + RW17C + AC3 ( $\geq 3,500 \text{ ng/ml}$ ) was always greater than B + RW17C ( $<1,000 \text{ ng/ml}$ ) or B + AC3 ( $<1,000 \text{ ng/ml}$ ) alone or the sum of the latter two



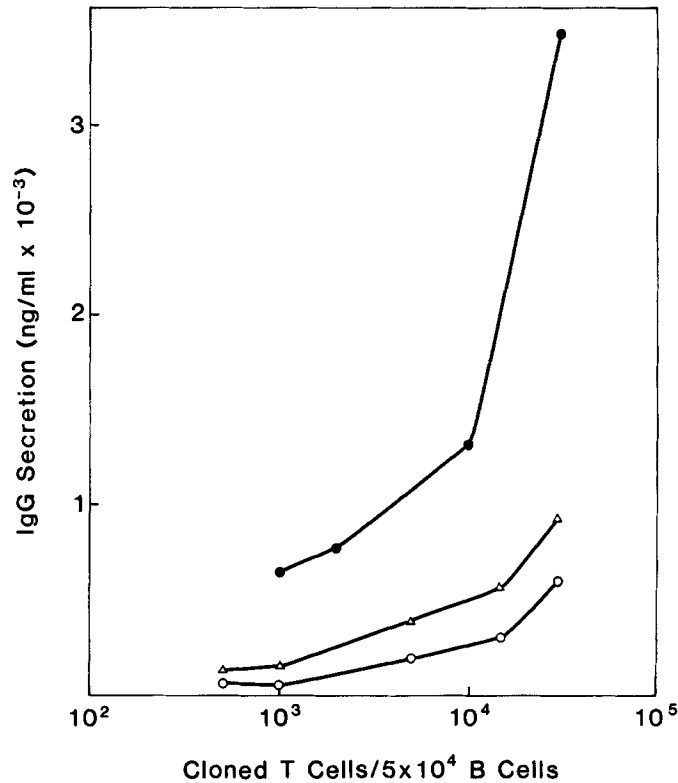


FIGURE 3. Amplifier effect of clone AC3 on IgG secretion induced by RW17C clone.  $5 \times 10^4$  autologous B cells and  $5 \mu\text{g/ml}$  RWAGE were cultured with: increasing numbers of either AC3 cells (○—○), RW17C cells ( $\Delta$ — $\Delta$ ), or a mixture of AC3 and RW17C (●—●). Note that the number of cloned T cells in this latter case represent the sum of AC3 and RW17C added in the assay. IgG secretion was measured on day 8 and the SD of each concentration was  $<5\%$ .

(<2,000 ng/ml). While the absolute amount of IgG (1–15  $\mu\text{g/ml}$ ) secreted during the 7-d culture period varied among each of six individual experiments, in all cases RW17C and AC3 each alone induced 25–30-fold increases in IgG secretion over base line B cell production with RWAGE, whereas the combination of RW17C plus AC3 provided  $\geq 100$ -fold increase in IgG secretion. These findings provide strong evidence to support the notion that RW17C and AC3 clones function in a synergistic manner either by inducing different B cell populations to secrete Ig or alternatively, by inducing more Ig from the same B cell.

*Surface Molecules on Autoreactive T Cell Clones.* From studies with alloreactive human T cell clones, it has been demonstrated that several surface molecules appear to be involved in antigen recognition by human T lymphocytes (8–10). These include the monomorphic 20/25-kD T3 structure that is present on all mature T lymphocytes and the subset-specific associative recognition elements, T4 and T8. More recently, Ti, a clonally unique receptor structure comprised of a 49–51-kD  $\alpha$  chain and a 43kD  $\beta$  chain, linked to T3 was identified on several of these clones and shown to co-modulate with the T3 structure.

To examine the important T cell surface structures required for recognition

of autologous MHC gene products, both antibody blocking studies and T3 modulation studies were performed. As shown in Table III, AC cells proliferate to IL-2-containing supernatant as well as the autologous B cell line Laz 509. Pretreatment of AC3 with purified anti-T3 or anti-T4 antibody markedly reduces antigen-specific proliferation of the AC3 clone. Thus, whereas the untreated AC3 clone proliferated with >80,000 counts of  $^3\text{H-TdR}$  to Laz 509, AC3 preincubated with anti-T3 or anti-T4 proliferated with <13,000 cpm, a >80% reduction of proliferation. That these effects were not simply due to inactivation or AC3 as a result of antibody treatment is clear from the fact that (a) anti-T8 (Table III) and anti-HLA (Fig. 1) have no inhibitory effects in this system and (b) the antibody treatment has no effect on subsequent IL-2 induced proliferation (Table III).

The above proliferative studies were performed in the presence of the purified monoclonal antibodies. Since the T3 structure has been previously demonstrated to modulate from the cell surface during incubation with anti-T3 antibody for 18 h at 37°C, it was possible to examine the proliferative responses of the AC3 population in the absence of the T3 molecule and the anti-T3 directed toward it. As shown in Table III B, AC3 no longer proliferates to Laz 509 (133 vs. >20,000 cpm) following anti-T3 modulation. In contrast, in the modulated state IL-2 responsiveness is markedly enhanced (>20,000 vs. 6,300 cpm), an observation noted previously for alloreactive CTL clones (9).

Recent studies indicated that the T<sub>i</sub> receptor molecules on individual T4+ and T8+ clones are contained within a molecular complex with T3 and can be detected in SDS-PAGE of anti-T3 immunoprecipitates from  $^{125}\text{I}$ -labeled alloreactive clones (29). To determine whether an analogous clonotypic structure was likewise found in the anti-T3 immunoprecipitate from AC3, immunoprecipitation experiments were performed with externally labeled  $^{125}\text{I}$  membrane preparations from AC3. As shown in Fig. 4, monoclonal anti-T3<sub>C</sub> precipitates

TABLE III  
*Influence of Monoclonal Anti-T Lymphocyte Antibodies on Proliferative Responses of Clone AC3*

A.	Antibodies in culture			
	Medium	Anti-T3	Anti-T4	Anti-T8
Medium	76 ± 16	93 ± 19	68 ± 20	92 ± 12
Laz 509	88,621 ± 2,557	11,477 ± 888	12,131 ± 894	95,915 ± 3,694
IL-2	58,042 ± 485	59,492 ± 1,138	47,756 ± 1,124	54,394 ± 550
B.	Anti-T3 modulation			
	Unmodulated	Modulated		
Medium	17 ± 3	21 ± 5		
Laz 509	20,569 ± 1,253	133 ± 10		
IL-2	6,339 ± 243	21,353 ± 1,018		

(A)  $3 \times 10^4$  cells of AC3 were cultured either in media, with  $5 \times 10^4$  irradiated autologous EBV transformed lymphoblastoid B cell line (Laz 509) or in 10% IL-2 in the presence or absence of each purified anti-T lymphocyte antibody (20 µg/ml). (B) Cells of clone AC3 were preincubated with anti-T3 antibodies for 18 h at 37°C, subsequently washed to remove free monoclonal antibody and then  $3 \times 10^4$  cells cultured with  $5 \times 10^4$  irradiated Laz 509 or 10% IL-2 as above. Unmodulated AC3 cells were preincubated with media. Results are expressed as cpm ± SD.

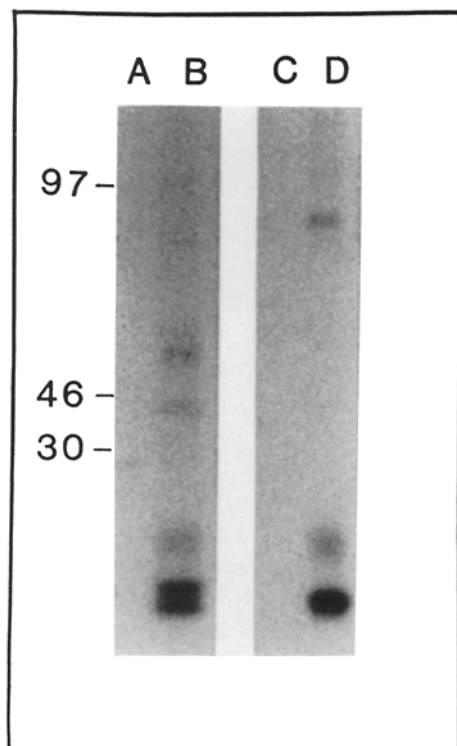


FIGURE 4. SDS-PAGE of anti-T3 immunoprecipitates from  $^{125}\text{I}$ -labeled AC3 clone. T-x-100 lysates from  $^{125}\text{I}$ -lactoperoxidase catalyzed surface labeling of AC3 were immunoprecipitated with anti-T3 monoclonal antibody as described in Materials and Methods. A, control (anti-T6), reduced; B, anti-T3, reduced; C, control, nonreduced; D, anti-T3, nonreduced.

four bands on SDS-PAGE under reducing conditions from AC3 (lane B): the major protein has a molecular weight of 20 kD with three additional bands at 25, 43, and 52 kD. More importantly, the two higher molecular weight bands correspond to the  $\alpha$  and  $\beta$  subunits of the T<sub>i</sub> structure that have been defined on other clones. Further support for this notion comes from the fact that under nonreducing conditions, the 52- and 43-kD upper bands detected by anti-T3<sub>c</sub> appear as a 90-kD structure (lane D). Note that the 20- and 25-kD subunits within the anti-T3 precipitate are similar under both reduced and nonreduced conditions. Taken together with the functional data, these results suggest that the T3 complex on alloreactive and autoreactive clones is analogous.

### Discussion

In the present study, peripheral blood mononuclear cells were stimulated by an autologous ragweed antigen-specific, MHC-restricted T4+ inducer clone termed RW17C and the resulting T cells clones were propagated in long-term *in vitro* culture ( $\geq 6$  months). 13 T4+ clones (AC1-13) were generated and found to be reactive with autologous class II but not class I MHC gene products, as defined by monoclonal antibody blocking studies and the unique specificity of AC1-13 for autologous but not allogeneic B lymphoblastoid line cells. Moreover,

the ability of different autologous peripheral blood cell types to serve as stimulators for AC1-13 proliferation appeared to correlate with their surface density of Ia molecules. Thus, Ia<sup>-</sup> autologous T cells were not stimulatory, whereas Ia<sup>+</sup> autologous B cells, macrophages, and B lymphoblastoid cells, in that order, induced increasing amounts of proliferation.

These same AC (i.e. AC3) induced autologous B cells to produce large amounts of immunoglobulin in the absence of exogenous antigen. In contrast, the immunizing clone RW17C induced autologous B cell Ig production only in the presence of the exogenous soluble antigen RWAGE. However, both sets of clones could trigger allogeneic B cells to produce Ig as long as autologous class II bearing cells (i.e. macrophages and in the case of RW17C, RWAGE) were present. The latter results imply that initiation of helper function from either type of clonal population is MHC restricted, whereas production of IgG can be induced from both autologous as well as allogeneic B cells. However, whether specific IgG production is MHC restricted is still to be resolved. Furthermore, the enhancement of Ig production from the combination of AC3 and RW17C cloned cells suggests that autoclones such as AC3 subserve a normal amplifier function. The finding that AC that appear after immunization recognize a "self" Ia molecule and are involved in amplifier function implies that they may be components of a critical immunoregulatory inducer circuit.

It is particularly interesting that the AC clones resulted from stimulation of PBMC with irradiated RW17C, yet were themselves only minimally able to proliferate to RW17C directly. These results suggested that RW17C served to trigger a second population to stimulate AC1-13 proliferation. Given that the AC clones are class II specific, it is possible that the latter was either a B cell or a macrophage. The inductive capacity of AC3 on B cell Ig production further supports this view.

Prior studies at the heterogeneous population level have provided support for the notion that amplifier populations for B and T cell responses exist in both man and mouse (30-32). For example, in the human system, T4<sup>+</sup> amplifier cells appear to preferentially express Ia antigen early in the course of activation. Thus, when soluble antigen-stimulated T cells were separated after 6 d of culture into T4<sup>+</sup>Ia<sup>+</sup> and T4<sup>+</sup>Ia<sup>-</sup> populations, it was found that the majority of proliferation was contained within the Ia<sup>+</sup> fraction and that most of this was nonspecific. More importantly, each of the T4<sup>+</sup> subsets alone induced B cell Ig production, but only together provided maximal induction of helper function (30). Although the present set of autoreactive T cells was generated from culture with RW17C, it is also clear that T cells reactive with self-MHC antigens can be generated by other means. In fact, when one immunizes with antigen-pulsed macrophages, one elicits clones specific for antigen and self-MHC as well as autoreactive clones directed at autologous MHC molecules (data not shown). Note that the polymorphic class II specificity of these and other clones and their growth in human serum argues against the possibility that such cells are directed at xenogeneic protein determinants, as suggested by others to explain apparent autoreactivity (33).

The present findings suggest that positive induction of immune responses in man may occur at either of two levels (Fig. 5). One level is soluble antigen

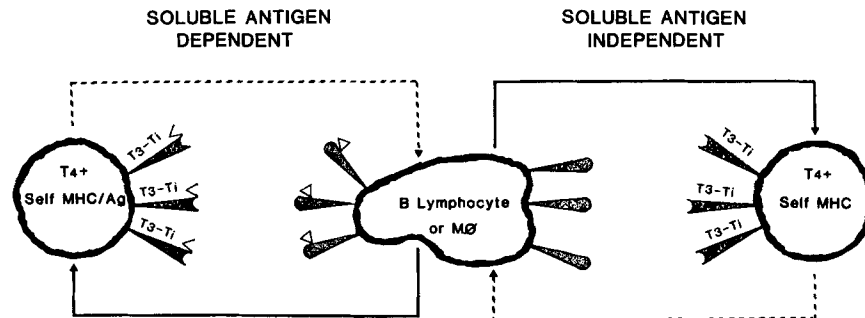


FIGURE 5. Model of soluble antigen-dependent and independent clonal T cell interaction. Soluble (foreign) antigen ( $\Delta$ ) in the context of autologous class II MHC ( $\text{C} \Rightarrow$ ) leads to activation ( $\rightarrow$ ) of Ag specific T4+ inducer T lymphocytes via their specific T3-Ti molecular complex which serves as receptor for both Ag and MHC. Subsequently, monocytes and/or B lymphocytes are activated ( $\dashrightarrow$ ) which results in triggering of autoreactive self-class II MHC specific ( $\rightarrow$ ) clones as a result of their own unique T3-Ti molecular complex. These soluble antigen independent (autoreactive) clones further activate B cells ( $\dashrightarrow$ ) and amplify the function of the soluble antigen dependent inducer cell.

dependent and MHC restricted as exemplified by RW17C, and the second is soluble antigen-independent and requires only autologous class II recognition. Such an antigen-“independent” level of immunoregulation is initiated by an internal set of signals (i.e. autologous MHC) and in conjunction with antigen-“dependent” inducer cells may serve to markedly amplify the immune response. This could be particularly important after antigen has been degraded and is no longer able to provide continued stimulation of the antigen-“dependent” level of immune induction.

The observation that RW17C induced little AC1-13 proliferation despite the fact that it expressed autologous Ia molecules is of considerable interest. While it is conceivable that the level of Ia expression on RW17C is simply too low to stimulate these clones to proliferate, one cannot rule out the possibility that a specific MHC class II gene product required for T cell stimulation is absent from the surface of RW17C. In this regard, it is well known that the class II MHC gene locus is complex and consists of at least three separate regions encoding for DR, DC, and SB (34). Molecular approaches to the problem including Northern blot analysis of T cell clone mRNA with cDNA probes to each of these regions will be important to study.

There is considerable evidence that endogenous synthesis and expression of Ia molecules occur as a consequence of T cell activation (35-37). Therefore, the possible failure of T cell “Ia” to induce secondary responses, as suggested by others (38), may be an important mechanism to limit further immune responsiveness by other T cells. While lack of stimulation by T cell Ia may have some direct regulatory effect, additional mechanisms yet to be defined and probably involving T8 suppressor cells generated during the autologous response are likely more important to control amplification mediated by clones such as AC1-13. Subsequent analysis of the T8+ populations generated during stimulation will be critical.

A model of human T cell antigen recognition has been proposed on the basis of functional and biochemical analysis of surface components on T lymphocytes

with specificities directed at allo class I or class II MHC gene products or viral gene products in the context of autologous MHC molecules. It was hypothesized that the T3-Ti molecular complex viewed polymorphic self-MHC plus antigen X (or polymorphic alloantigen alone), whereas T4 and T8 served as ancillary associative recognition structures for monomorphic regions of class II or class I molecules, respectively (39). Several independent lines of evidence suggest that T3-Ti is the T cell receptor: (a) both  $\alpha$  and  $\beta$  subunits of Ti molecules from clones of differing specificities (but derived from the same donor) contain clonally unique peptides on two-dimensional peptide map analysis (40) and (b) anti-T3 or the appropriate anti-Ti antibody covalently cross-linked to Sepharose is, by itself, capable of triggering clonal proliferation and lymphokine production analogous to the combination of antigen and MHC restricting elements (41).

In contrast, T4 and T8 do not appear to be specific antigen receptor molecules. Sepharose-bound anti-T4 and anti-T8 antibodies do not trigger clonal proliferation and there is no biochemical evidence to suggest that that structures that they define are polymorphic at the peptide level. Nevertheless, T4 and T8 appear to be extremely important in facilitating cell-cell interactions by, presumably, providing additional affinity for ligand binding in conjunction with T3-Ti molecular complex. That this is the case is clearly evident from the observation that soluble anti-T4 and anti-T8 antibodies are capable of blocking killing by CTL directed at class II and class I MHC gene products, respectively on target cells. (1, 2, 4, 43).

The present study supports the view that autoreactive clones like AC3 recognize a polymorphic region of a class II molecules since it proliferated to autologous but not allogeneic Ia. The observation that anti-T3 modulation abrogates this proliferative response implies that the recognition of autologous polymorphic regions of MHC gene occurs via T3-Ti and that T4 is not the singular recognition site for class II MHC gene products. Moreover, the ability of purified anti-T4 antibody to block the AC3 proliferation induced by autologous class II molecules further supports the notion that T4 may facilitate binding of class II molecules to T cells, particularly if T3-Ti binding to auto-MHC is low affinity.

It is of interest that earlier studies with class II allospecific CTL in general demonstrated profound blocking of cytotoxicity but only partial, if any, blocking of alloantigen-induced proliferation by anti-T4 (9). Given the likelihood that there is a more stringent requirement for cell-cell contact in the former response, this differential effect is not surprising. The rather complete blocking by anti-T4 of autoreactive AC3 proliferation to autologous MHC class II gene products contrasts with the minimal effects of anti-T4 on alloreactive clonal proliferation reported previously (9). It is possible that this difference may be related to the lower affinity of self-MHC-specific T3-Ti complexes in comparison to allo-MHC-specific T3-Ti complexes.

While we have yet to prepare a monoclonal antibody to the clonotype of one or another AC, the biochemical evidence to date indicates that the T3-Ti molecular complex of AC3 is strikingly similar to T3-Ti molecular complexes of alloreactive CTL in terms of their component subunits (29). Thus, anti-T3 could precipitate in addition to 20/25-kD structures, 43-kD, and 52-kD components analogous to those found on allogeneic clones. Moreover, under nonreducing

conditions, the 43- and 52-kD components appeared as a 90-kD band consistent with the notion that the clonotypic structure on AC3 was a disulfide-linked heterodimer, as previously found on "conventional" alloreactive and MHC-restricted/antigen-specific T cell clones (9). These findings imply that T3-Ti is essential for all forms of antigen recognition, even those restricted to autologous MHC gene products. Presumably some T3-Ti complexes view antigen X and self-MHC (i.e. T3-Ti from RW17C), others view self-MHC alone (i.e. AC3) and yet others view antigen X alone. Perhaps an example of the "antigen X alone" clone is the recently described natural killer (NK)-like T cell clone, which clearly had a broad but definite specificity unrelated to MHC antigens (44).

Although we have defined no clones in the present study with unique specificity for the stimulating clone RW17C, others have demonstrated such specific clone anti-clone responses (16). Thus, it would seem possible that some T3-Ti molecular complexes could be directed at autologous T3-Ti structures on clones of differing specificities. The latter might account for regulation at the clonal level.

The autoreactive nature of AC1-13, their specificity for class II MHC gene products, T4+ subset derivation, and lack of cytotoxic effector functions are characteristics that have been described for cells proliferating in autologous mixed lymphocyte reaction (AMLR) in human peripheral blood (11-15, 17). Presumably, AC1-13 cells represent the clonal counterpart of the AMLR responsive cell in peripheral blood. These studies suggest that such cells subserve an amplifier function in T cell regulation of the normal immune response. Given the existence of such autoreactive cells in healthy donors and the ease with which they can be elicited, their existence argues against the concept of clonal deletion in self tolerance. Understanding of the regulation of the AC response now becomes important as it may provide further insights into both physiologic and pathologic immune functions, including autoimmunity.

### Summary

Autoreactive T lymphocytes were generated by culturing human peripheral blood mononuclear cells with an antigen-specific major histocompatibility complex (MHC)-restricted autologous inducer T cell, termed RW17C and subsequently cloned in soft agar. The majority of such clones (AC1-13) expressed the T3+T4+T8-T11+Ia+ phenotype and were directed at autologous class II MHC gene products found on B cells, macrophages, and B lymphoblastoid cells as judged by their proliferative response to the latter. For this recognition, the clones employed a T3-Ti molecular complex and a T4 structure analogous to those found on allospecific T cells. Perhaps more importantly, it was observed that the same AC1-13 autoreactive clones (AC) induced autologous B cells to produce high levels of immunoglobulin in the absence of exogenous antigen and could synergize with the RW17C clone to effect maximal B cell Ig production. These results support the notion that such autoreactive cells can function in a physiologic amplifier role by facilitating induction via an internal set of signals (i.e. autologous MHC).

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