IMMUNOGLOBULIN SECRETION IN THE HUMAN AUTOLOGOUS MIXED LEUKOCYTE REACTION

Definition of a Suppressor-Amplifier Circuit Using Monoclonal Antibodies*

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The regulation of the immune system in experimental animals, including the production of immunoglobulin (Ig) by B cells, is dependent in large part on interactions among T cell subsets, each with its own special function and distinguishable from other T cells on the basis of differences in surface antigens (1). Studies of animal models (2-4) have led more recently to the discovery of immunoregulatory networks of communicating, phenotypically different T cells, each providing a distinct step in a circuit leading to the inhibition of an immune response. Such circuits have been termed feedback suppression (2, 4) or suppressor amplification (5). Germain and Benacerraf (6) have elegantly synthesized several of the better known murine suppressor networks into a single major pathway of T lymphocyte interactions. Within this synthesis it is possible to distinguish an Lyt-1 suppressor-inducer cell that helps in the activation of a precursor suppressor-amplifier cell, (generally Lyt-1,23) to a suppressor-amplifier cell, which in turn activates a suppressor-effector, Lyt-23 cell.

In humans, the recent production of monoclonal antibodies (7, 8) to human T cell subsets has enabled the demonstration that Ig synthesis by B cells is also subject to T cell-mediated regulation (9-12). However, there have been only limited studies of immunoregulatory circuits in man (13-15). This report uses the generation of Ig synthesis in the autologous mixed leukocyte reaction (MLR)[1] to expand this work and provide details of a suppressor-amplifier circuit.

The autologous MLR is generally defined as T cell proliferation after an autologous Ia$^+$, non-T stimulus (16). In our hands (12) and others (17), however, the autologous MLR has proved a useful model to study the regulation of immunoglobulin synthesis in man. Using previously described monoclonal antibodies (8, 11, 12) that allow the division of peripheral blood T cells into nonoverlapping subsets, Leu-3 (helper/inducer) cells and Leu-2 (suppressor/cytotoxic) cells, we showed that the induction of Ig synthesis in the autologous MLR has an absolute requirement for Leu-3 cells,

whereas an excess of Leu-2 cells suppress the response (12). The current study was undertaken to further assess the immunoregulatory potential of T cells activated in the autologous MLR. To this end, cells activated in a primary autologous MLR were separated into subsets using monoclonal antibodies to T cell markers (8, 11, 12) and HLA-DR antigen (18). The results demonstrate that activated Leu-2,DR$^+$ T cells, but neither Leu-2,DR$^-$ nor Leu-3 T cells, can act in a second autologous MLR as potent, radioresistant suppressor-amplifier cells of Ig synthesis. The activation of this subpopulation requires Leu-3 cells in the primary culture; furthermore, in the absence of fresh Leu-2 cells in the second culture, little or no suppression was observed. These results suggest that at least two distinct subpopulations of Leu-2 cells are required for optimum suppression of the immune response and that immunoregulatory circuits analogous to those described in the mouse (2-6) exist in man.

Materials and Methods

Monoclonal Antibodies.   The monoclonal antibodies we used were produced by the method of somatic cell hybridization (19). The T cell subset monoclonals, anti-Leu-2a and anti-Leu-3a, were generously provided by Dr. R. L. Evans, Memorial Sloan-Kettering Cancer Center, New York, and have been described previously (8, 11, 12). In the present study, the antibodies were used as tissue culture supernatants of cloned cell lines.

The monoclonal antibodies CA77, 135, 141, 206, and BM50, originally prepared by Dr. D. J. Charron as partially purified ascites fluids (18), were used in certain experiments. These antibodies, all of which fix complement, are directed against nonpolymorphic determinants of HLA-D (Ia) molecules (18) and bind to B lymphocytes, most monocytes, but <3% of normal resting peripheral T cells (20).

Isolation of E-rosetting and Nonrosetting Cells from Peripheral Blood.   Peripheral blood mononuclear leukocytes (PBML) were obtained from normal human volunteers by Ficoll-Hypaque gradient centrifugation (21). Populations enriched for T and non-T cells were prepared by a single step rosetting method (22) using 2-aminoethylisothiouronium bromide hydrobromide (Sigma Chemical Co., St. Louis, MO) -treated sheep erythrocytes (SRBC) and a second Ficoll-Hypaque gradient to separate the rosetted T cells from the nonrosetted (non-T) cells. The rosetted cells were separated from the SRBC by hypotonic lysis of the latter and an aliquot set aside for assessment of purity as has been previously described (12).

Isolation of Mononuclear Phagocytes.   After culture in some experiments, plastic-adherent and nonadherent cells were separated before panning according to the method of Hausman et al. (23). The nonadherent fraction contained <1% alpha naphthyl acetate esterase (ANAE)-positive cells, and the adherent fraction >95% ANAE-positive cells (12).

Isolation of T Cell Subsets.   Purified subsets of T cells were obtained by a panning technique (24) which permitted the fractionation of fresh or cultured T cells into Leu-2$^+$/Leu-2$^-$ or Leu-3$^+$/Leu-3$^-$ subpopulations (11, 12). The purity of both positively and negatively selected fractions was tested using indirect immunofluorescence and fluorescence-activated cell sorter (FACS) analysis (25). If anti-Leu-2 was used as the first stage incubating antibody, then <2% of the unbound cells were Leu-2$^+$ and >95% of the bound cells were Leu-2$^+$. Similar subset purity was achieved when anti-Leu-3 was used as the first stage incubating antibody. In a series of preliminary experiments, all separation procedures were carried out with heat-inactivated autologous serum instead of fetal calf serum.

Monoclonal anti-DR, either CA141 or CA206, were used in a similar fashion at a dilution of 1:100 to further subdivide Leu-3$^-$ (Leu-2) cells after four washings of the latter. Analysis with FACS again confirmed the purity of separated subsets.

Pretreatment of Cells with Anti-DR Plus Complement.   In certain experiments, Leu-3$^-$ (Leu-2) cells were depleted of DR$^+$ cells by incubation with a pool of anti-DR monoclonals; CA77, 135, 141, 206, BM50 (18) plus complement as described by Engleman et al. (20).

Cell Cultures.   Primary autologous MLR were performed in 25-cm^2 tissue culture flasks (25100; Corning Glass Works, Corning, NY) in RPMI 1640 medium supplemented with 25 mM Hepes buffer, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20%

heat-inactivated autologous or pooled human serum. Each flask contained 10^7 irradiated (2,500 rad,[137]Cesium unit; Shepherd JL and Assoc., Glendale, CA) non-T stimulators and 2×10^7 T cell or T cell subset responders in 15 ml of medium. After a period of culture from 1 to 12 d in humidified 10% CO_2 in air, the activated T cells were harvested, various subsets prepared as described above, and added to autologous MLR, which were cultured in microwells (12). On the day of harvest, cells from several wells were pooled, washed three times in balanced salt solution (BSS), counted, and resuspended in 0.8 ml of BSS in preparation for the assay for plaque-forming cells (PFC).

Protein A PFC Assay. A polyclonal plaque-forming assay was used to detect IgM- and IgG-secreting B cells exactly as has been described by us previously (12). Geometric means and the standard deviation of the number of PFC generated by 10^6 nonrosetting cells originally placed into culture were used to express the data. The percentage of suppression was calculated by the formula: $(1 - [(\text{suppressor cells} + \text{control cells})/(\text{control cells alone})]) \times 100$.

Results

Effect of Activated Leu-2 Cells on Fresh Autologous MLR. Cells were cultured in bulk autologous MLR for 8 d, after which the Leu-2 cells were separated by panning and added in the ratios shown (Fig. 1) to fresh autologous cultures containing 10^5 non-T cells and 2×10^5 T or Leu-3 cells per microwell. In the typical example illustrated after a further 8-d culture, activated Leu-2 cells added to fresh T cells at a ratio of 1:10 resulted in 89% and 78% suppression of the IgM- and IgG-PFC, respectively; and at a ratio of 1:50, activated Leu-2 cells produced 88% and 54% suppression, respectively, when added to cultures containing total T cells. Also shown in Fig. 1, when these same low numbers of activated Leu-2 cells were added to cultures containing no fresh Leu-2 cells, no significant suppression was seen. This pattern was repeated in eight consecutive experiments with cells from five unrelated individuals using either autologous or pooled human serum. At ratios of 1:1 activated Leu-2 cells to fresh Leu-3 cells, ~50–80% suppression of the PFC response was observed (data not shown), an effect similar to that reported previously for fresh autologous Leu-2 cells (12). In contrast, the suppressor effect of the preactivated Leu-2 cells in the presence of fresh Leu-2 cells was at least 50 times more potent than fresh Leu-2 cells alone (12). In experiments not shown, if these preactivated Leu-2 cells were added to fresh cultures that had been underway for 72 h, no suppression was seen.
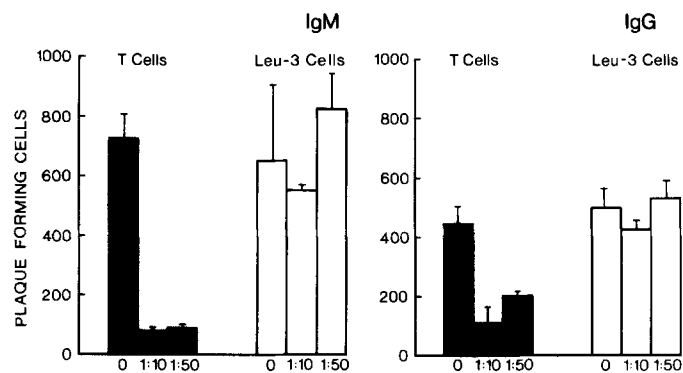

FIG. 1. Suppressive effect of activated Leu-2 cells on Ig production in the autologous MLR. Horizontal axes show the ratio of activated Leu-2 cells to fresh T or Leu-3 cells in culture. Vertical columns show the mean and standard deviation of the PFC generated per 10^6 non-T cells originally placed in fresh microwell culture.

Effect of Leu-3 Cells on the Activation of Leu-2 Suppressor-Amplifier Cells.   To determine if helper T cells play a role in the activation of Leu-2 suppressor-amplifier cells, bulk cultures were initiated with either 2×10^7 Leu-2 cells and 10^7 non-T cells per flask or 2×10^7 unseparated T cells and 10^7 non-T cells per flask. After 9 d culture, in the example illustrated (Fig. 2), Leu-2 cells were separated from both types of culture and added to fresh cultures in ratios of 1:1, 1:10, and 1:50, and PFC assayed after 8 d microwell culture. At ratios of 1:1, both populations of preactivated Leu-2 cells resulted in suppression of the PFC response. However, at ratios of 1:10 and 1:50, only Leu-2 cells activated in the presence of Leu-3 cells produced suppression of the IgM and IgG-PFC. Leu-2 cells obtained from primary cultures that lacked Leu-3 cells were no more inhibitory than fresh Leu-2 cells.

Kinetics of Activation of Leu-2 Suppressor-Amplifier Cells.   To determine the time required for the development of suppressor-amplifier cells, Leu-2 cells were isolated after varying periods of culture in autologous MLR and tested for the capacity to suppress PFC induction in fresh autologous MLR (Fig. 3). In the example shown, little or no suppressor-amplifier activity was detected before 4 d of culture. Maximum suppression of both IgM and IgG-PFC developed after 8–10 d of primary culture and remained relatively constant thenceforth.

Kinetics of the Suppressive Effects of Activated Leu-2 Cells.   In the experiments described above, PFC were determined at the usual time point of peak response in autologous MLR-induced Ig synthesis (12). To assess the possibility that activated Leu-2 cells might alter kinetics of this response, Leu-2 cells were isolated from 8-d autologous MLR and added to fresh microwell cultures containing 10^5 non-T and 2×10^5 T or Leu-3 cells per well (Fig. 4). The results of IgM- and IgG-PFC generation show suppression of Ig synthesis throughout the second culture period with no evidence of altered kinetics of response. By contrast, secondary cultures lacking fresh Leu-2 cells were not inhibited by activated Leu-2 cells, regardless of the time of harvest.

DR Phenotype of the Suppressor-Amplifier Leu-2 Cells.   T cells activated with a variety of stimuli have been shown to express HLA-DR antigen (26–28). After autologous
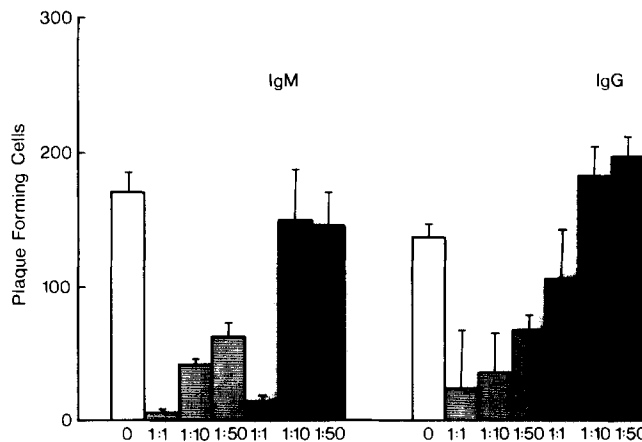

Fig. 2.   Leu-3 cells are required for activation of Leu-2 suppressor-amplifier cells. Horizontal axes show the ratio of Leu-2 cells activated in the presence (▤) or absence (■) of Leu-3 cells in fresh autologous culture. Vertical columns show the mean and standard deviation of the PFC generated per 10^5 non-T cells originally placed in fresh microwell culture.
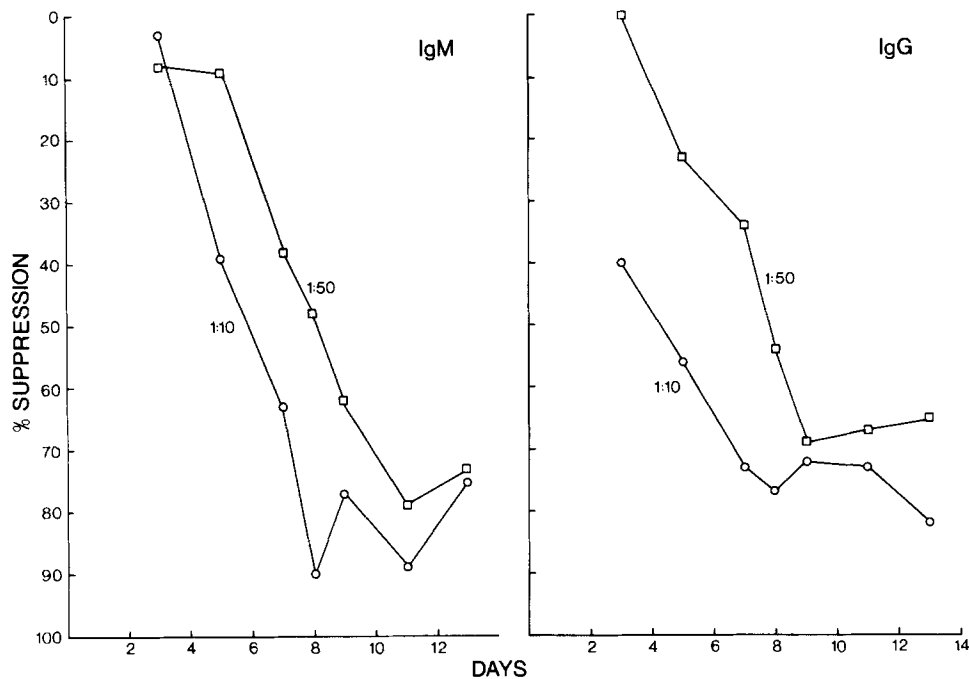
Fig. 3. Kinetics of activation of Leu-2 suppressor-amplifier cells. The vertical axis shows the percent suppression calculated as described in Materials and Methods. The horizontal axis is the number of days of culture of the primary auto MLR before separation of the activated Leu-2 cells. The ratios, 1:10 (O) and 1:50 (☐), are the proportion of activated Leu-2 cells to fresh T cells in the microwell cultures.

MLR, at least 20% of T cells from both Leu-2 and Leu-3 subsets are DR positive (C. J. Benike and E. G. Engleman, unpublished observations). To determine whether the expression of DR antigen on activated Leu-2 cells correlated with suppression, Leu-3⁻ cells were isolated by panning from 8-d autologous MLR. After treatment with monoclonal anti-DR, these cells were subdivided into Leu-3⁻, DR⁺ and Leu-3⁻, DR⁻ cells, then added to fresh microwell cultures in the ratios shown (Fig. 5), and PFC assayed after 8 d further culture. Although at 1:1 ratios of activated cells to fresh cells in both the Leu-2, DR⁺ and Leu-2, DR⁻, cells produce suppression similar to that seen in primary cultures (12), at the low ratios of 1:10 and 1:50 the suppressive activity was confined to the Leu-2, DR⁺ fraction.

As the activated Leu-3⁻ (Leu-2⁺) cells could have contained mononuclear phagocytes, these experiments were performed with a preadherence step, which left <1% ANAE-positive cells. The purified mononuclear phagocytes were tested in microwell cultures and showed no suppressive effects when 2 × 10⁴ of such activated macrophages were added to each well (data not shown). Similar results were obtained in four experiments in three different individuals. Pretreatment of the Leu-2 cells with pooled monoclonal anti-DR and complement had no effect on the ability of activated Leu-2 cells to suppress PFC generation in a fresh autologous MLR (data not shown).

Effect of Gamma Irradiation of Preactivated and Fresh Leu-2 Cells. It was of interest to determine whether proliferation of suppressor-amplifier cells (Leu-2, DR⁺) or the suppressor-effector cells (Leu-2, DR⁻) is required for their respective effects. After 8 d,
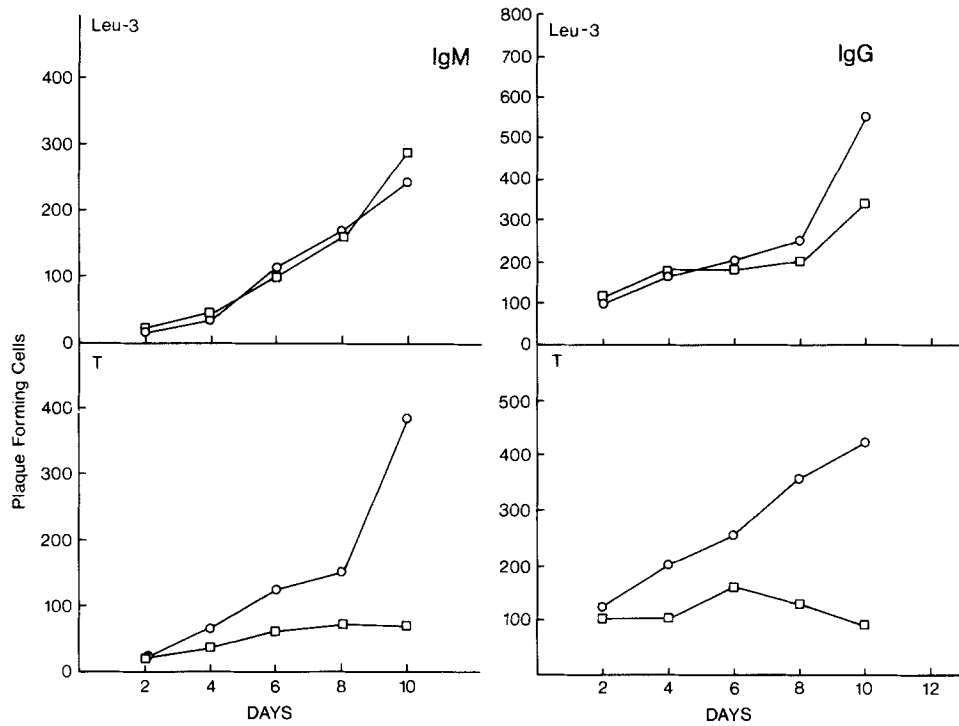
Fig. 4.   Kinetics of the suppressor effects of activated Leu-2 cells. The source of fresh helper cells in this experiment is labeled on the four panels of the figure. The horizontal axes show the time in days after initiation of the microwell cultures. The vertical axes show the number of PFC per 10^6 non-T cells originally placed in microwell culture. Each point represents the mean number of PFC generated at a given time of harvest. The error bars are omitted for clarity; the standard deviations were <15% of the response. Control cultures are indicated (O). Cultures containing activated Leu-2 cells in a 1:10 ratio to either fresh T or Leu-3 cells are indicated (□).

bulk culture-activated Leu-2 cells were separated by panning, and 2×10^4 of these cells were added to each well of fresh cultures containing 2×10^5 Leu-3 cells, 10^5 Leu-2 cells, and 10^5 non-T cells per well. As shown in the example (Fig. 6), either the preactivated Leu-2 cells, the fresh Leu-2 cells, or both were irradiated to 1,500 rad. PFC were assayed after 8 d of culture. The results show that maximum suppression is seen when both fresh and preactivated Leu-2 cells were unirradiated. Irradiation of the fresh Leu-2 cells removed suppression, but irradiation of the preactivated Leu-2 cells alone had little or no effect on suppression. Irradiation of both Leu-2 sets completely ablated suppression.

Effect of Preactivated Leu-3 Cells on Fresh Autologous MLR.   In a series of preliminary experiments, it was observed that preactivated Leu-3 cells, when added to fresh autologous MLR, lead to a reduction in the usual peak of Ig synthesis. To examine the possibility that the addition of activated Leu-3 cells may alter the time or magnitude of the peak PFC response, a kinetic study was performed. T cells were activated in primary flask cultures, and the Leu-3 cells were separated after 8 d. These cells were added in the ratios shown (Fig. 7) to fresh autologous MLR containing 2×10^5 T or Leu-3 cells and 10^5 non-T cells per well. In the typical case illustrated, preactivated Leu-3 cells lead to an earlier and in some cases higher peak of Ig synthesis
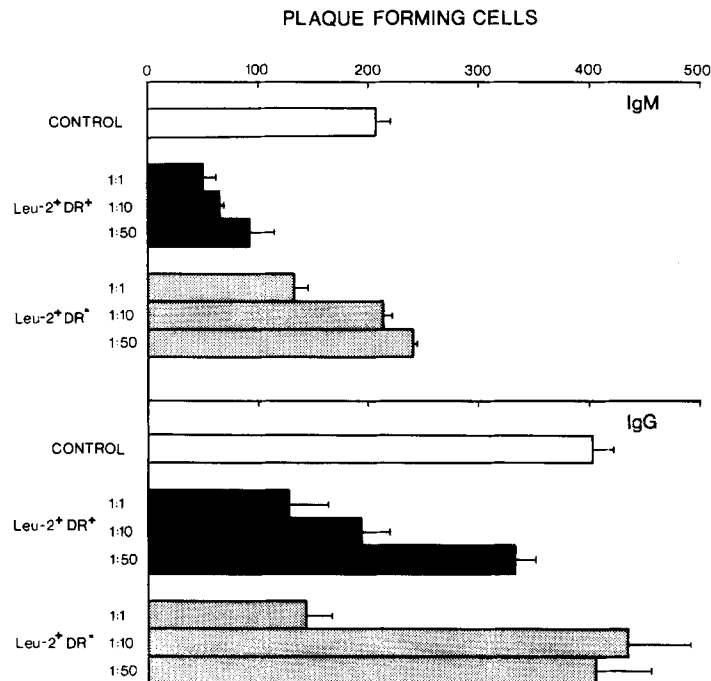
PLAQUE FORMING CELLS

Fig. 5. Leu-2 suppressor amplifier cells express HLA-DR. The columns represent the mean number of PFC and standard deviation generated in each group per 10^6 non-T cells originally placed in fresh microwell culture.

followed by a fall with apparent suppression of the PFC response by the time maximum antibody synthesis was reached in control cultures. This same pattern was seen in four different individuals.

Discussion

The autologous MLR has proved a useful reaction in studies of immunoregulation in man, and although its physiological role remains uncertain, it demonstrates the typical attributes of an immune response, memory, and specificity (16). In this study, the use of a double culture system, whereby T cells or T cell subsets are preactivated in autologous MLR and then tested in a second autologous MLR, has allowed the characterization of cells involved in an immunoregulatory circuit in man. The schema illustrated in Fig. 8 demonstrates the cells that have been defined and their surface phenotypes characterized so far. Thus, we have produced evidence of a Leu-3 cell necessary for the activation of a Leu-2 cell, which initiates the suppressor circuit (Fig. 2). This cell can be designated a suppressor-inducer cell and appears to subserve a similar function to an Lyt-1 cell described in various antigen-specific murine systems (2-6). Previous results from other groups using pokeweed mitogen (PWM) (10) and antigen-driven (14) systems in humans have produced evidence of the need for helper T cells to induce suppressor activity within the reciprocal suppressor population. We have previously shown that Leu-2 cells alone react poorly in the autologous MLR, and it is not surprising that Leu-3 cells are required for the activation of Leu-2 cells; indeed, in the presence of Leu-3 cells, Leu-2 cells proliferate quite adequately (11).
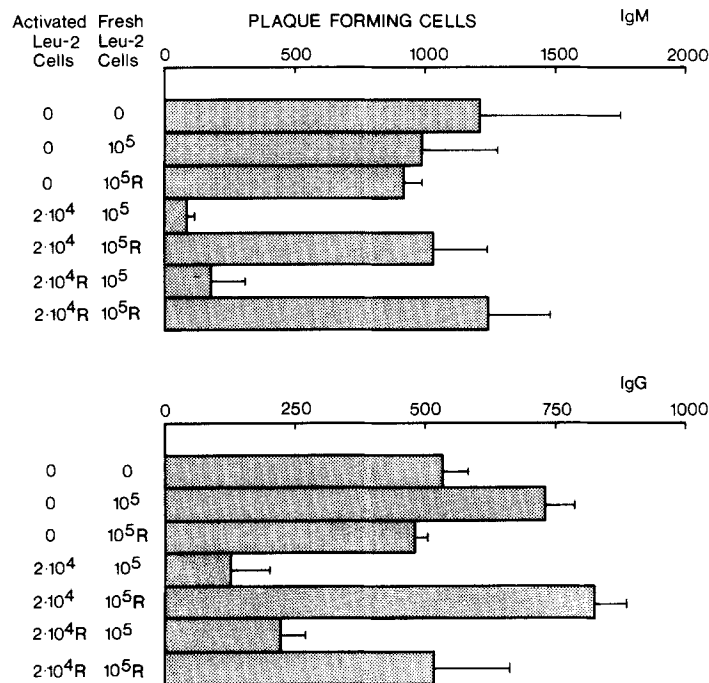
Fig. 6.   Effect of gamma irradiation on activated suppressor-amplifier and fresh suppressor-effector Leu-2 cells. On the left, adjacent to each column, are the number of preactivated Leu-2 cells and fresh Leu-2 cells in each microwell of the second culture. The columns represent the mean number of PFC and standard deviation generated in each group per 10^6 non-T cells originally placed in microwell culture (R = 1,500 rad).

The suppressor-amplifier precursor cell defined in these experiments (Fig. 8) bears the Leu-2 phenotype, but is negative for DR antigens as defined by the pool of monoclonal antibodies used in this study. This cell appears analogous to the murine suppressor-amplifier precursor as summarized by Germain and Benacerraf (6). The suppressor-amplifier cell characterized in these experiments resembles the feedback-suppressor cell defined in the mouse (2–6). The cell has the phenotype Leu-2, DR$^+$ (Fig. 5) and demonstrates a potent, radioresistant recruiting effect on fresh Leu-2 cells (Figs. 1, 6), which it can presumably perform without the need to undergo cell division. Within the fresh Leu-2 population, however, the radiosensitive suppressor-effector cells appear to have to undergo cell division to exert their optimum effect.

The suppressor-amplifier cell appears to arise from an initially DR-negative cell population and develops DR antigens on its surface as it becomes activated. Such optimal activation indeed takes 8–10 d (Fig. 3), a period during which DR antigens may appear on the surface of an originally negative population. The development of Ia or DR antigens on the surface of T cells has been associated with the expression of specific function, including the ability to generate helper activity for Ig synthesis (29) and to suppress proliferation induced by alloantigens (28) or mitogens (30). The presence of Ia or DR antigens on a cell involved in suppression is in contrast to the absence of Ia on human cytotoxic T cells (31).

The third cell in this feedback-suppressor network is a Leu-2 suppressor-effector cell, which acts to inhibit the generation of antibody-forming cells (Fig. 6). The
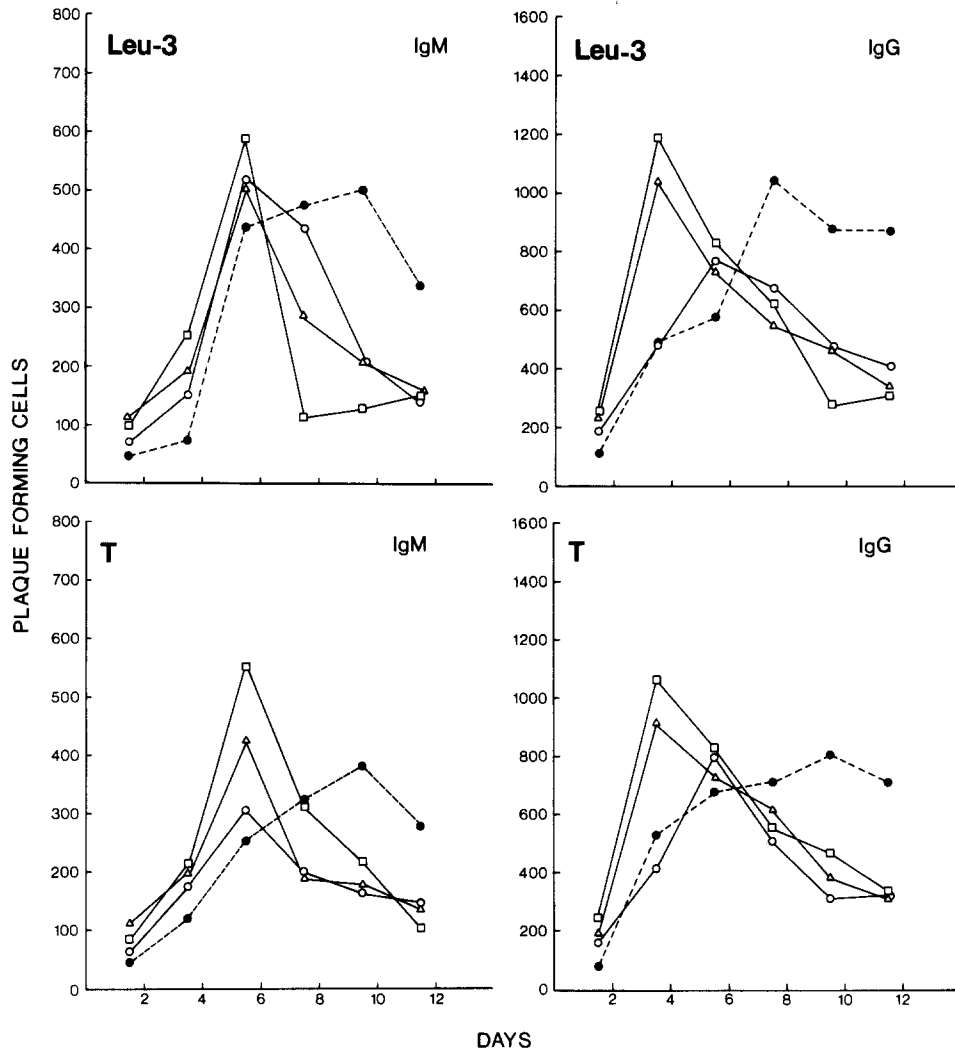
Fig. 7.   Effect of activated Leu-3 cells in fresh autologous MLR. The horizontal axes show the time in days after initiation of the microwell cultures. The vertical axes show the number of PFC per 10^6 non-T cells originally placed in fresh microwell culture. Each point represents the mean number of PFC generated at a given time of harvest. The error bars are omitted for clarity. The standard deviations were <15% of the response. Control cultures are indicated (●). The ratios 1:10 (□), 1:20 (△), and 1:50 (○) refer to the proportion per well of preactivated Leu-3 cells to either fresh T or Leu-3 cells.

sensitivity of this cell to gamma irradiation suggests that it has to divide, as we have previously demonstrated (12) for Leu-2-mediated suppression of Ig synthesis in the primary autologous MLR. However, although division must occur, the combined inhibitor effect of the amplifier and effector-suppressor cells on PFC generation in fresh autologous MLR is detected as soon as 4 d after initiation of culture (Fig. 3), suggesting that early cellular interactions determine the suppressed state. This interpretation is supported by the observation that addition of activated Leu-2 cells to 3-d-old cultures fails to suppress Ig synthesis.
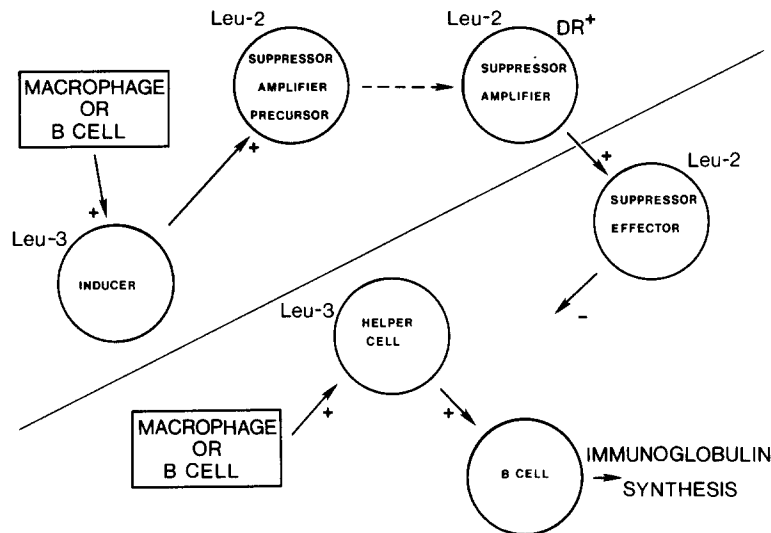
Leu-2

Leu-2 DR$^+$

SUPPRESSOR AMPLIFIER PRECURSOR

SUPPRESSOR AMPLIFIER

MACROPHAGE OR B CELL

Leu-2

SUPPRESSOR EFFECTOR

Leu-3

INDUCER

Leu-3 HELPER CELL

MACROPHAGE OR B CELL

B CELL

IMMUNOGLOBULIN SYNTHESIS

Fig. 8. Schema of the immunoregulatory interactions investigated in this study. →+ or − indicate putative positive and negative signals, respectively. → indicates a step which may represent the action of one cell type upon another, or the maturation of one cell type into the other. The surface phenotype defined is marked on each cell type. The events envisaged occurring in the first culture are shown above, those in the second culture, below.

This suppressor-effector cell described here again shows analogy to the Lyt-23 suppressor-effector cell of murine systems (2-6). In summary, therefore, these experiments have produced evidence for Leu-3-dependent activation of a Leu-2 suppressor-amplifier precursor cell, which once activated expresses DR antigen, and although at low cell numbers it exerts little or no suppression itself, can act as a potent radioresistant suppressor-amplifier cell that can activate fresh Leu-2 suppressor-effector cells to inhibit Ig synthesis generated in an autologous MLR.

Initial studies of the effects of precultured Leu-3 cells on the generation of PFC in the autologous MLR suggested that these cells also exerted an inhibitory effect. However, kinetic analysis of the Leu-3 effect demonstrated an early peak of Ig synthesis followed by a fall below the peak response of the controls. This effect was seen even in experiments in which there were no Leu-2 cells present in the second microwell culture. Although a suppressor mechanism cannot be formally ruled out, the effect seen could have a simple kinetic explanation with consumption of important factors or nutrients concomitant with the early peak of Ig synthesis in the cultures to which preactivated Leu-3 cells were added. Suppression by activated cells of helper phenotype (10) has been described in a PWM-driven stimulation of Ig synthesis (13, 32). Although the system used in these studies and the generation of Ig synthesis in the autologous MLR described here are not strictly comparable, it is significant that no kinetic studies were reported (13, 32).

The experiments reported here raise the question of further definition of abnormalities of the autologous MLR in disease. The proliferative response to an autologous non-T stimulus is defective in NZB/W mice, a murine model for systemic lupus erythematosus, as well as several other strains associated with autoimmune syndromes (33). In the NZB/W mouse model, there is a deficiency of feedback suppressor, or

suppressor-amplifier cells (33). Additional studies of the generation of suppressor-amplifier cells in the autologous MLR of patients with systemic lupus erythematosus, a disease in which a defective autologous MLR has been reported (34, 35), and in other autoimmune diseases should help shed light upon the details of their immune abnormalities.

Summary

The induction of immunoglobulin (Ig) synthesis in the autologous MLR has an absolute requirement for helper/inducer (Leu-3) T cells, whereas an excess of suppressor/cytotoxic (Leu-2) cells suppresses the response. The current study was an effort to assess the immunoregulatory potential of T cells activated in the autologous mixed-leukocyte response (MLR). T cells were cultured with autologous non-T cells for 8–9 d, after which the activated T cells were fractionated into subsets with monoclonal antibodies to T cell markers and HLA-DR antigen. Each population was co-cultured in fresh autologous MLR, and on the 8th day of culture, Ig-secreting cells were measured in a reverse hemolytic plaque assay. The results show that activated Leu-2, DR^+ T cells, but neither Leu-2, DR^- nor Leu-3 T cells, were at least 50 times more potent as suppressors of IgM and IgG synthesis than fresh Leu-2 cells alone. The activation of this Leu-2, DR^+ subpopulation required Leu-3 cells in the primary culture. Furthermore, in the absence of Leu-2 cells in the second culture, little or no suppression was observed, suggesting that the Leu-2, DR^+ cells act to amplify or induce suppressor effects of fresh Leu-2 cells. This indicates that at least two distinct subpopulations of Leu-2 cells are required for maximal suppression of an immune response, and that immunoregulatory circuits analogous to those described in the mouse exist in man.

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