INTERLEUKIN 4 CAUSES ISOYPE SWITCHING TO IgE IN T CELL-STIMULATED CLONAL B CELL CULTURES

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IL-4 has been shown to enhance both IgG1 and IgE synthesis in bulk cultures of LPS-stimulated B cells (1-4). Analysis of the IL-4 requirements for both IgG1 and IgE production has suggested that there may be differences in the mechanism by which IL-4 regulates these isotypes (5). A major unresolved issue is whether IL-4 mediates specific heavy chain class switching to these isotypes or preferentially allows for the growth and maturation of cells already committed to the expression of these isotypes. To address this issue, limiting dilution analyses of LPS-stimulated B cells have been conducted and have shown that IL-4 increases the frequency of precursors that give rise to IgG1 (6, 7) or to IgE-secreting cells (8). Although these studies suggest that IL-4 mediates isotype switching, definitive proof of the mechanism of action of IL-4 requires the establishment of clonal cultures in which the isotypes secreted by an individual precursor can be ascertained.

In this paper, we describe a culture system that allows a majority of B cells to be stimulated in a T cell–and antigen-dependent fashion. This culture system takes advantage of the observation that B cells efficiently process and present rabbit antibodies directed against mouse Ig to T cells specific for rabbit gamma globulin (9). Isotype analysis of the supernatants of clonal cultures demonstrates that stimulation with an IL-4-producing Th2 cell line (10) causes the majority of precursor cells to undergo heavy chain class switching during clonal expansion. To inhibit the effects of IL-4 produced by the Th2 cells, a blocking mAb directed against IL-4 (11) was added to some cultures. In the presence of anti-IL-4, there was considerable decrease in both the proportion of precursor cells that gave rise to clones secreting IgE and the amount of IgE secreted by those clones. Thus, IL-4 acts by specifically causing heavy chain class switching to IgE. However, anti-IL-4 has no effect on the proportion of clones that secrete IgG1, which suggests that other consequences of the interaction between Th2 cells and B cells play a role in the generation of an IgG1 response.

Materials and Methods

Animals. Female (C3H x DBA/2)F1 mice were purchased from The Jackson Laboratories, Bar Harbor, ME, and were used between 6-12 wk of age.

Lymphokines. Murine rIL-4 expressed in Escherichia coli and purified to >95% purity was supplied by Schering Research, Bloomfield, NJ. The bio-assay to determine IL-4 activity was performed as described (10). 1 U of IL-4 is defined as the amount in 1 ml that gives one-half maximal stimulation in the HT-2 assay (10). rIL-4 was used at 60 U/ml.

Antibodies. The preparation and specificity of rabbit antibodies directed against mouse IgM, IgG1, and IgE have been previously described (12). Rabbit anti-mouse κ antibodies
were prepared from the sera of rabbits immunized with mouse monoclonal IgG2a, IgE, or IgA proteins and were eluted from an immunoabsorbent column of J606 (IgG3, κ) coupled to Affi-gel-10 (Bio-Rad Laboratories, Richmond, CA). The eluates were pooled and passed through a rat Ig Affi-gel column. The anti-IL-4 and anti-IgD antibodies were mAbs 11B11 (11) and 10.4.22 (13), respectively. Anti-IL-4 was added to the cultures at 5 μg/ml.

**Cell Preparation.** Single cell suspensions of spleen were depleted of T cells by treatment with anti-Thy-1 mAb 30H12 (14) followed by Low-Tox guinea pig complement (Accurate Chemical Co., San Diego, CA). Live cells were separated from dead cells and erythrocytes on Histopaque (density = 1.119; Sigma Chemical Co., St. Louis, MO). The T-depleted population obtained in this fashion was ~90% B cells. To obtain slgM\(^+\), slgD\(^-\) B cells, spleen cell suspensions were stained with nitroiodophenyl acetic acid (NIP)\(^-\)-conjugated rabbit anti-mouse IgM (RAM IgM) and biotin-conjugated 10.4.22 followed by FITC-conjugated anti-NIP and phycoerythrin-conjugated streptavidin (Becton Dickinson & Co., Mountain View, CA). The slgM\(^+\), slgD\(^-\) population was separated on the basis of fluorescence using a FACS IV (Becton Dickinson & Co.).

**T Cell Line.** The Th cell line CDC25 was the generous gift of David Parker, University of Massachusetts Medical School, Worcester, MA. This cell line is specific for the F(ab')\(_2\) fragment of rabbit IgG in the context of a hybrid I-A\(^{k}\) determinant (9). Upon stimulation with Con A, CDC25 secretes IL-4 and IL-5 and is a member of the Th2 subset of helper cells (15).

**Microculture Protocol.** Clonal cultures were establishment in Terasaki trays (Nunc, Roskilde, Denmark) in 10 μl of RPMI 1640 supplemented with 10% heat inactivated FCS, 4 mM L-glutamine, 100 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 1% HBI02 supplement (Hana Biologics, Berkeley, CA) and maintained in an atmosphere of 5% CO\(_2\) and 90% humidity. Each culture contained limiting numbers of B cells and 2 × 10\(^3\) resting (8-10 d after stimulation) CDC25 cells that had been irradiated with 1,000 rad \(\gamma\) irradiation. The development of clones required the presence of antigen, 5 ng/ml RAM IgM. 10 d after the initiation of cultures, supernatants were harvested and screened in an ELISA (12) for Ig secretion using rabbit anti-mouse κ. Positive wells were assayed for the presence of IgM, IgG1, and IgE using an isotype-specific ELISA (12). Cultures to which anti-IL-4 had been added could not be screened with rabbit anti-mouse κ because 11B11 antibody molecules contain mouse κ chains. These cultures were all assayed for the presence of IgM, IgG1, and IgE. Only cultures with a 90% or greater probability of arising from a single precursor, as determined by Poisson analysis (16), were reported.

**Statistics.** The culture conditions used were determined to conform to a single-hit Poisson distribution using the maximum likelihood calculation (17). Thus, when 20% or fewer of the culture wells are positive, there is at least a 90% probability of clonality. To achieve clonality B cells were plated out at 0.25-0.5 cells/well. Only cultures with a 90% or greater probability of clonality are reported. Fisher's test was used to determine the significance of the effect of anti-IL-4 on the proportion of clones secreting IgE. The significance of the difference in IgE secretion by individual clones generated in the presence of different relative amounts of IL-4 was determined by the Wilcoxon rank sum test.

**Results**

**A High Proportion of B Cells Respond to Th2 Stimulation.** To ascertain if the culture conditions conformed to a single-hit Poisson distribution in which the B cells were limiting, B cells were plated at a concentration of 0.125-5 cells/well into wells containing 2 × 10\(^3\) irradiated CDC25 T cells and RAM IgM. Antibody secretion and clonal expansion were not observed unless both T cells and antigen were present; omission of either resulted in no clones. Fig. 1 demonstrates that these culture conditions followed a single-hit Poisson distribution. Approximately one-half of the cells

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1 Abbreviations used in this paper: NIP, nitroiodophenyl acetic acid; RAM, rabbit anti-mouse.
in a T-depleted cell population were capable of responding in these culture conditions. Thus, when 20% or fewer of the culture wells are positive, there is a 90% probability that each positive well arises from a single precursor. In each individual experiment, three different concentrations of B cells were used in order to confirm that the particular experiment followed a Poisson distribution. To achieve a high probability of clonality, the cultures analyzed for secretion of individual isotypes were derived from wells to which B cells had been added at a concentration of 0.25–0.5 cells/well.

**IL-4 Causes Isotype Switching to IgE.** The effect of IL-4 on the isotypes secreted by individual clones was evaluated by varying the concentration of IL-4 in the cultures. CDC25 secretes IL-4 and IL-5 when activated (15), but based on results obtained in bulk cultures, the level of IL-4 in these microcultures could be limiting for IgE responses. To vary the IL-4 concentration, either rIL-4 at 60 U/ml or anti-IL-4 at 5 μg/ml was added to cultures. The addition of 5 μg/ml anti-IL-4 has been shown to cause a 500-fold reduction in the amount of detectable IL-4 in supernatants of Con A–stimulated Th2 cells (15) and it completely inhibits IgE production in bulk cultures of B cells stimulated with Th2 cells (18). In such cultures, the addition of 60 U/ml IL-4 stimulates optimal IgE secretion, usually enhancing the amount of IgE secreted by 2–10-fold (Coffman, R. L., B. Seymour, H. Cherwinski, J. Christiansen, D. Parker, and T. Mosman, submitted for publication). Stimulation with CDC25 Th cells in the presence of RAMIgM caused a high proportion of B cells to secrete IgG1 and/or IgE during clonal expansion. Clones did not develop unless antigen was present. The addition of exogenous IL-4 had little effect on the proportion of clones secreting IgG1 or IgE. However, the addition of anti-IL-4 to cultures substantially reduced the proportion of clones that secreted IgE, 78% in the presence of exogenous IL-4 compared with 33% in the presence of anti-IL-4 (Table I).
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**Table I**

*IL-4 Increases the Proportion of Clones that Secrete IgE*

| Isotypes expressed          | Percent clones expressing with added: |
|-----------------------------|-------------------------------------|
|                             | Medium* | IL-4† | anti-IL-4‡ |
| IgM, no IgG1 or IgE         | 10      | 5     | 19         |
| IgG1, no IgM or IgE         | 10      | 12    | 16         |
| IgE, no IgM or IgG1         | 3       | 7     | 12         |
| IgM + IgG1                  | 13      | 5     | 32         |
| IgM + IgG1 + IgE            | 22      | 26    | 12         |
| IgG1 + IgE                  | 39      | 45    | 9          |
| IgM + IgE                   | 3       | 0     | 0          |
| IgE +/- IgM, IgG1           | 68      | 78    | 33         |
| IgG1 +/- IgM, IgE           | 84      | 88    | 70         |
| IgM +/- IgG1, IgE           | 48      | 36    | 63         |

The difference in the proportion of clones secreting IgE in the presence of IL-4 compared with anti-IL-4: \( p = 1.9 \times 10^{-3} \).

* B cells were stimulated with CDC25 in the presence of 5 ng/ml RAM IgM. 31 clones analyzed.
† 60 U/ml of rIL-4 was added to cultures. 42 clones analyzed.
‡ 5 μg/ml 11B11 was added to cultures. 43 clones analyzed.

Furthermore, the addition of exogenous IL-4 results in a decrease in the proportion of clones that secrete IgM (36%) when compared with the addition of anti-IL-4 (63%). The data presented in Table I are pooled from two experiments in which there was an equally high probability of clonality under all three tested conditions. In five individual experiments, considering only clonal cultures, 0-48% of the clones derived from anti-IL-4-treated cultures secreted IgE, whereas 72-90% of those derived from cultures with added IL-4 secreted IgE. In contrast to the effect of anti-IL-4 on the proportion of clones that secrete IgE, the addition of anti-IL-4 does not change the proportion of clones that secrete IgG1. Between 85-100% of cultures that secreted detectable levels of Ig could be isotyped as secreting IgM, IgG1, IgE, or a combination of these isotypes. Thus, the number of clones secreting at least one of these three isotypes approximately equaled the number of responding precursors and was regarded as the number of clones analyzed. When supernatants from 60 cultures, each of which contained at least one precursor, were pooled and the levels of all secreted isotypes were measured (IgM, IgG1, IgE, IgG2a, IgG2b, IgG3, and IgA), the sum of the IgM, IgG1, and IgE responses constituted >95% of the total Ig secreted in all three conditions under which clonal cultures were established (data not shown).

The ability of anti-IL-4 to inhibit the proportion of clones secreting IgE varied among experiments. However, when compared with the addition of anti-IL-4, the addition of IL-4 consistently increased the amount of IgE secreted by individual clones. The data shown in Fig. 2 is derived from an experiment in which 48% of the clones generated in the presence of anti-IL-4 secreted IgE compared with 92% and 80% of the clones generated either with additional IL-4, or with no additions (medium), respectively. The addition of exogenous IL-4 caused approximately an eightfold increase in the average amount of IgE secreted by an individual clone compared with cultures containing anti-IL-4. There is approximately a threefold increase in average IgG1 secretion when these two culture conditions are compared (data not shown).
FIGURE 2. IL-4 increases the amount of IgE secreted by individual clones. Each point represents one IgE-secreting clone. A horizontal line is placed at the mean. Medium: B cells were stimulated with CDC25 in the presence of 5 ng/ml RAMIgM; 80% of the clones generated secreted IgE. IL-4: 60 U/ml rIL-4 was added to cultures; 92% of the clones generated secreted IgE. Anti-IL-4: 5 ng/ml 11B11 was added to the cultures; 48% of the clones generated secreted IgE. The statistical significance of these results is: Medium vs. anti-IL-4, p = 0.02; Medium vs. IL-4, p = 0.11; anti-IL-4 vs. IL-4, p = 0.002.

IgE-secreting Clones Are Derived from sIgM+, sIgD+ Precursors. In the experiments described previously, the antigen used was RAMIgM, which strongly suggests that the precursors of these clones expressed sIgM. To confirm that the IgE-secreting clones were derived from precursors that expressed sIgM, the effect of IL-4 on the isotypes secreted by clones derived from FACS-purified sIgM+, sIgD+ B cells was determined. Table II shows that the isotypes secreted by this population in response

| Isotypes expressed | Percent of clones expressing with added: |  
|-------------------|------------------------------------------|
|                   | Anti-IL-4* | IL-4† |
| IgM, no IgG1 or IgE | 19 | 0 |
| IgG1, no IgM or IgE | 7 | 6 |
| IgE, no IgM or IgG1 | 0 | 17 |
| IgM + IgG1 | 44 | 11 |
| IgM + IgG1 + IgE | 26 | 33 |
| IgG1 + IgE | 4 | 33 |
| IgE +/− IgM, IgG1 | 30 | 83 |
| IgG1 +/− IgM, IgE | 81 | 83 |
| IgM +/− IgG1, IgE | 89 | 44 |

Frequency: 0.51 0.65

The difference in the proportion of clones secreting IgE: p = 4.7 x 10^{-4}.  
* B cells were stimulated with CDC25 and 5 ng/ml RAM IgM. Cultures received 5 μg/ml 11B11. 27 clones analyzed.  
† Cultures received 60 U/ml of rIL-4. 18 clones analyzed.  
§ Frequency is the fraction of responsive cells determined by dividing the number of precursors by the number of input cells.
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to IL-4 and anti-IL-4 are comparable to those secreted by the unfractionated population (compare Table I). In particular, in the presence of anti-IL-4 a greater proportion of clones secrete IgM (89 vs. 44%) and a smaller proportion of clones secrete IgE (30 vs. 83%). The proportion of cells that respond to stimulation and undergo clonal expression is comparable in both conditions.

Discussion

The enhancement by IL-4 of both IgG1 and IgE production in LPS-stimulated B cells has been well documented (1-5). However, these studies have not clearly demonstrated whether IL-4 enhances the frequency of switching to these isotypes. To gain insight into the mechanism of IL-4 action, we have established a clonal culture system in which the effects of IL-4 on isotype secretion can be determined. This system is based on the observations that B cells can efficiently process and present rabbit antibodies directed against mouse surface Ig to T cells specific for rabbit gamma globulin (9). It has been shown that, at the appropriate concentrations, rabbit anti-mouse Ig and specific antigen are comparable in their abilities to initiate T-B interactions (19). Thus, by using RAMIgM as an antigen, it is possible to polyclonally stimulate B cells that are sIgM+ by a T cell- and antigen-dependent mechanism. This microculture system has several advantages over previously described systems (20, 21). Most important is the fact that a majority of B cells can respond in a physiologically relevant manner without the need for exogenous filler cells or poorly defined supernatants. Since in this system every B cells is, in principle, both an antigen-binding and an antigen-presenting cell, no special techniques for the enrichment of antigen-specific B cells are required. This permits the analysis of B cells whose responses are not affected by antigen binding or by other manipulations that occur before stimulation. Thus, this microculture system can be used to determine if IL-4 alters isotype secretion patterns during Th-dependent, antigen-dependent clonal expansion.

Limiting dilution analyses of LPS-stimulated B cell cultures have shown that IL-4 increases the frequency of precursor cells that give rise to IgG1- (6, 7) and IgE- (8) secreting cells. However, since these studies did not establish a direct precursor/product relationship, they did not definitively demonstrate whether IL-4 acts to increase the frequency of isotype switching to IgG1 or IgE or whether it allows preferential outgrowth of precommitted precursors. To vary the concentration of IL-4, a neutralizing antibody to IL-4 was added to some cultures and an optimum concentration of rIL-4 was added to others. In bulk cultures stimulated by the CDC25 line, the addition of 60 U/ml rIL-4 gives maximum enhancement of the IgE response and 5 µg/ml of anti-IL-4 gives complete inhibition of the IgE response (Coffman, R. L., et al., submitted for publication). This suggests that the IL-4 concentration in bulk cultures can support substantial but not optimum IgE secretion, and this appears to be the case in clonal cultures as well. A comparison of cultures with added IL-4 and those with added anti-IL-4 demonstrates that the proportion of clones that secrete IgE increases with increasing IL-4 concentration. The addition of IL-4 substantially increases both the fraction of clones that secrete IgE and the amount of IgE secreted by these clones when compared with clones generated in the presence of anti-IL-4. Importantly, this occurs under conditions in which a majority of B cells give rise to clones and in which the frequency of responsive cells is not substan-
tially affected by adding or removing IL-4. Furthermore, the use of RAMIgM antibody as the "antigen" demonstrates that the IgE-producing clones initially expressed sIgM and, as shown using a FACS-separated population, most of the precursor cells expressed both sIgM and sIgD. Thus, these experiments definitively demonstrate that IL-4 acts to substantially increase the frequency with which individual B cells, activated by Th cells and antigen, switch from IgM to IgE production.

In addition to an increased frequency of clones that give rise to IgE-producing cells, the average level of IgE secreted by individual clones is significantly enhanced by the addition of IL-4. This could result from either an increase in the number of IgE-secreting cells contained in a clone or an increase in the amount of IgE produced by individual IgE-secreting cells. The former would be consistent with an IL-4-mediated increase in the frequency with which daughter cells could switch to IgE. The latter would suggest that IL-4 can also act as a selective growth or differentiation factor for sIgE+ cells. This latter activity would also have to be isotype specific since IL-4 does not cause a comparable increase in the amount of IgG1 or IgM secreted by individual clones. It should be noted that there is a relatively high proportion of clones that secrete IgE even in the presence of anti-IL-4. Although anti-IL-4 can substantially reduce the concentration of IL-4 in culture supernatants, it is possible that it cannot completely prevent B cells from coming into contact with IL-4 released as a consequence of T-B interaction and conjugate formation (22). Thus, the addition of anti-IL-4 can reduce the effective concentration of IL-4, but it can not completely remove IL-4 from the cultures.

In contrast to its ability to induce isotype switching to IgE, IL-4 does not appear to enhance isotype switching to IgG1. The proportion of precursors that gives rise to IgG1-secreting clones is the same regardless of the presence or absence of IL-4. Although this appears inconsistent with the enhancing effects of IL-4 on IgG1 production in LPS-stimulated cultures (3-5), it is consistent with other, more recent results using T cell-dependent cultures (18, 23). The addition of anti-IL-4 to bulk cultures of splenic B cells stimulated with a Th2 cell line and antigen completely abolishes the IgE response (18), but the IgG1 and IgM responses are, at most, only partially inhibited (18, 23). In addition, in bulk cultures, stimulation of B cells with Th1 cells that do not secrete IL-4 results in a substantial IgG1 response (18). These results suggest that substantial IgG1 responses can be obtained in these systems with little or no IL-4 present. A similar conclusion comes from in vivo studies (24). Mice injected with goat anti-mouse IgD have markedly increased levels of serum IgG1 and IgE. The IgE, but not the IgG1 response of these mice can be largely inhibited by the injection of anti-IL-4. Thus, the results obtained in clonal cultures are consistent both with results obtained in vitro in bulk cultures of Th2-stimulated B cells (18, 23) and in vivo (24) and suggest that IgG1 regulation in Th-stimulated cultures may be quite different than in LPS-stimulated cultures.

Since sIgM+ splenic B cell populations have been shown to contain few cells with µ-gene deletions (25) and since the majority of sIgM+ B cells respond in the clonal microcultures, it is likely that the switch to IgE involves IL-4-induced DNA rearrangements rather than the preferential outgrowth of B cells that had switched to IgE in vivo but had retained sufficient mRNA to remain sIgM+. In LPS-stimulated B cells, IL-4 induces a substantial increase in sIgG1+ B cells and the majority of these cells have Cy1 gene rearrangements and Cµ gene deletions (25).
High concentrations of IL-4 have also been shown to induce significant numbers of B cells bearing either IgE or IgG1 + IgE (26). One possible interpretation of this latter population is that IgE-producing cells do not arise directly from sIgM* cells, but rather by switching first to IgG1 and then to IgE. Our observations that IgM + IgE secreting clones rarely occur and that the majority of IgE-secreting clones also secrete IgG1 would be consistent with such a mechanism.

Limiting dilution analyses of polyclonally activated B cells in both humans (27, 28) and mice (8) have suggested that precursors of IgE-secreting cells represent a small subpopulation of B cells. B cell stimulation in these systems did not involve a Th-dependent, antigen-dependent mechanism analogous to that in our microcultures. Furthermore, the frequency of responsive cells is much lower than that obtained in the system we describe. Nonetheless, the level of IgE secreted by individual IgE-secreting clones (8, 27) is comparable to that secreted by clones stimulated with Th2 cells and RAMIgM. The low frequency of IgE-secreting precursors in these systems could reflect the properties of the B cell population that is stimulated and the mechanism of stimulation rather than limitations in the potential of most B cells to switch to IgE. Our experiments demonstrate that under the proper conditions the majority of sIgM-expressing B cells can be induced to give rise to IgE-secreting cells during clonal expansion. Further support for the concept that sIgM-expressing cells are not restricted in their potential to secrete particular isotypes such as IgE comes from analyses of antigen-specific clones generated in splenic fragment assays (29, 30). Isotype analysis of IgE-secreting clones showed that clones secreting IgE also secreted additional isotypes and there was no preferential association of IgE secretion with any other isotype. Thus, the isotypes secreted during clonal expansion appear to be a function of the way in which a B cell is stimulated rather than an intrinsic property of the B cell.

Summary

Although it has been established that IL-4 enhances both IgG1 and IgE secretion in LPS-stimulated B cell cultures, these studies failed to determine whether IL-4 preferentially induces isotype switching or preferentially allows for the maturation of precommitted precursor cells. To distinguish between these possibilities, it is necessary to ascertain the effect of IL-4 on the isotypes secreted by individual precursor cells during clonal expansion. Therefore, clonal cultures of B cells stimulated with a Th2 helper cell line specific for rabbit Ig and rabbit anti-mouse IgM were established. The majority of B cells are capable of undergoing clonal expansion under these conditions. To vary the level of IL-4 present, either IL-4 or anti-IL-4 was added to cultures. In the presence of IL-4 there was an increase in the proportion of clones that secreted IgE and a decrease in the proportion of clones that secreted IgM. The addition of IL-4 to cultures also increased the amount of IgE secreted by individual clones. Thus, these experiments definitively prove that IL-4 causes specific heavy chain class switching to IgE in Th2-stimulated B cell cultures. In contrast, IL-4 does not affect the proportion of clones secreting IgG1, suggesting that other consequences of Th cell-B cell interactions play a role in the generation of an IgG1 response.

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