ISOTYPE-SPECIFIC IMMUNOREGULATION

IgA-binding Factors Produced by Fca Receptor-positive T Cell Hybridomas Regulate IgA Responses

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Fc receptors (FcR)

Fc receptors (FcR)\(^1\), membrane glycoproteins which bind Ig molecules via their Fc region, have been identified (1) on the surface of various cell types. For example, FcR for IgG are expressed on macrophages, B and T lymphocytes, cytotoxic T lymphocytes, and natural killer cells. FcR are important in immune regulation, and several studies have addressed their role in isotype-specific responses. In this regard, T lymphocyte subpopulations have been described with FcR for IgM (Fc\(\gamma R\)) (2), IgG (Fc\(\delta R\)) (3), IgG (Fc\(\gamma R\)) (4), IgE (Fc\(\varepsilon R\)) (5), and IgA (Fc\(\alpha R\)) (6). Previous studies (1, 7, 8) have shown that Fc\(\gamma R\)-bearing (Fc\(\gamma R^+\)) T cells and T cell hybridomas regulate IgG production. These T cells release Fc\(\gamma R\) molecules, termed IgG-binding factors (IBF\(\gamma\)) that suppress IgG synthesis (7, 8). Others (9–12) have demonstrated that IgE-binding factors (IBF\(\varepsilon\)) released from Fc\(\varepsilon R^+\) T lymphocytes regulate IgE immune responses. IBF\(\varepsilon\) possess opposite regulatory functions; one factor specifically enhances the IgE response, while a second factor suppresses this isotype (10, 11). Both IgE-potentiating factor and IgE-suppressive factor are produced by the same Fc\(\alpha R^+\) T cell subpopulation (9, 12).

T lymphocytes bearing Fc\(\alpha R\) have been described (6, 13) in both humans and mice. Fc\(\alpha R^+\) T cells appear to be involved in regulation, because increased numbers of Fc\(\alpha R^+\) T lymphocytes of a suppressor phenotype have been isolated from mice with IgA myeloma (14), and have been shown (15) to suppress IgA isotype-specific responses. Others (16, 17) have reported that Fc\(\alpha R^+\) T cell hybridomas, or concanavalin A (Con A)-activated Fc\(\alpha R^+\) T cells, incubated with IgA, release IBF\(\alpha\) that can suppress IgA production in pokeweed mitogen-stimulated spleen cell cultures. On the other hand, Fc\(\alpha R^+\) T lymphocytes

\(^1\) Abbreviations used in this paper: Con A, concanavalin A; FcR, Fc receptor; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IBF, Ig-binding factor; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PFC, plaque-forming cell; PP, Peyer’s patch; PP Th A, Peyer’s patch T helper cell that supports IgA response; sIg, surface Ig; SRBC, sheep red blood cell; Th, T helper cell; Th HA, T helper cell hybridoma that supports IgA response; TNP, trimetaphen.
separated from human peripheral blood have been shown (18) to specifically enhance IgA responses in pokeweed mitogen–driven B cell cultures.

Our previous studies (19, 20) have shown that oral immunization of mice with the T cell–dependent antigen, sheep red blood cells (SRBC), induces significant T helper (Th) cell activity in Peyer’s patches (PP), and that this T cell population preferentially supports IgA immune responses both in vitro and in vivo. Subsequently, (21, 22) we have isolated and characterized Th cell clones from murine PP that express FcaR on their surface, and which largely support in vitro IgA responses (PP Th A cells). The preferential help for IgA responses was found to be mediated by collaboration between FcaR+ PP Th A cells and mature, IgA-committed B cells (22). On the other hand, T cell clones have been isolated from murine PP that appear to induce switching of surface IgM–positive (sIgM+) B cells to those expressing surface IgA (sIgA+) (23, 24).

In the present study, we have developed T–T hybridomas by fusing PP Th A cells with R1.1 T lymphoma cells to examine the molecular mechanisms involved in Th–B cell collaboration for IgA isotype–specific responses. These hybridomas have been maintained in fetal calf serum (FCS)-free medium. A number of hybridomas express FcaR, and supernatants from these cells regulate in vitro IgA responses. Characterization of these hybridomas and their released IBFa are discussed from the standpoint of their role in selection, triggering, and regulation of committed sIgA+ B cells for IgA isotype synthesis.

Materials and Methods

**T Cell Lines.** PP Th A cells (Thy-1.2+, Lyt-1+, FcaR+, and H-2k) were derived from PP of C3H/HeJ (H-2k) mice, as previously described (21, 22). Two types of PP Th A cells were used for the production of T–T hybridomas: PP Th A clone 1 showed some support for IgM responses, in addition to its strong support of IgA responses; PP Th A clone 9 showed low-level support for IgM and for IgG subclasses, and greatly elevated IgA responses (21). The R1.1 T lymphoma cell line used for cell fusion expresses Thy-1.2, TL, and H-2k (25, 26) and was kindly provided by Dr. J. F. Kearney (Department of Microbiology, University of Alabama at Birmingham).

**Formation of T-T Hybridomas.** For the generation of hybridomas, PP Th A clones 1 or 9 were fused with R1.1 T lymphoma cells using polyethylene glycol, mol wt 4,000 (Sigma Chemical Co., St. Louis, MO), as previously described (8, 27, 28). Equal numbers of PP Th A cells and R1.1 cells were pelleted together and fused by adding 1 ml of 50% polyethylene glycol in RPMI 1640 (Gibco Laboratories, Grand Island, NY). After 1 min of incubation at 37°C, the cell suspension was gradually diluted to 10 ml with RPMI 1640 over a 5 min interval. The cells were washed by centrifugation (1,000 rpm) in RPMI 1640. After washing, cells were resuspended in medium consisting of complete medium (RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 x 10⁻⁵ M 2-mercaptoethanol and 10% FCS) with 0.01 M hypoxanthine, 4 x 10⁻⁷ M aminopterin, and 1.6 x 10⁻⁶ M thymidine (28). After 3–4 wk of growth in selective medium, it was gradually removed and replaced by complete medium.

After growth of hybrid cells in complete medium (3–4 wk), culture supernatants were screened by the in vitro plaque-forming cell (PFC) assay (see below). Briefly, supernatant fluids were added to lipopolysaccharide (LPS)-stimulated splenic B cell cultures, and immunized with SRBC. After 5 d of incubation, cells were harvested and tested for IgA anti-SRBC PFC responses. Individual wells of hybridomas that produced factors for the enhancement of in vitro IgA immune responses were subcloned by limiting dilution. Selected subclones were expanded and maintained in culture with complete medium. Additional sets of selected subclones were gradually adapted to growth in FCS-free medium, HB 102 (Hana Biologics, Berkeley, CA). Cells were first cultured in a mixture
of 50% HB 102 and 50% complete medium, then in 90% HB 102 and 10% complete medium before being shifted to FCS-free medium.

Characterization of T-T Hybridomas. Cell suspensions (1–2 × 10⁶ cells) were incubated (45 min, 4°C) with 20 μl of fluorescein isothiocyanate (FITC)-labeled anti-Thy-1.2 (clone 30 H-12), anti-Lyt-1 (clone 53-7.313), anti-Lyt-2 (clone 53-6-72), or anti-I-Ak (clone 11-5.2) monoclonal antibody (mAb). The cells were then washed three times with phosphate-buffered saline (PBS) containing 1% FCS. The proportion of cells that stained with FITC-labeled reagent was determined by counting ≥ 1,000 cells using a fluorescence microscope (Orthoplan, Leitz, Inc., Wetzlar, Federal Republic of Germany).

An immunocytoadherence assay was performed for assessment of surface Fc receptors on T-T hybridomas. Trinitrophenyl (TNP)-sensitized SRBC were prepared as previously described (21, 29). MOPC 315 IgA against dinitrophenyl (DNP) was purified by DNP affinity chromatography (30). IgM (H5-3) and IgG1 (H35-3) anti-DNP mAb were kindly provided by J. O. Phillips (Department of Microbiology, University of Alabama at Birmingham). Hybridoma cells (4 × 10⁶ cells/ml) were treated with 100–200 μg/ml of anti-DNP mAb for 1 h at 37°C, then washed three times with RPMI 1640 containing 2% FCS. The mAb-treated cells were mixed with a 0.5% suspension of TNP-SRBC and incubated for 30 min at 4°C. Rosette-forming cells were scored by the immunocytoadherence assay (21).

Splenic and PP Cell Preparations. Spleens and PP were aseptically removed from C3H/HeN (H-2k) mice that were either left untreated, or were orally primed with SRBC. Single cell suspensions were prepared as previously described (19, 20). Spleen cells were gently passed through sterile stainless steel screens into the appropriate media. Cells from PP were obtained by disassociating the tissue with the enzyme Dispase in Joklik-modified medium (Gibco Laboratories) (19, 20).

Preparation of B and T Lymphocytes, and Accessory Cells. Splenic or PP single-cell suspensions from normal mice were treated with a cocktail of anti-T cell mAb (anti-mouse Thy-1.2 [clone H0-13-4], anti-Lyt-1 [clone 53-7.313], and anti-Lyt-2 [clone 53-6-72]) as described previously (20, 22). Cells were washed in minimal essential medium (Gibco Laboratories) supplemented with L-glutamine, gentamicin, sodium bicarbonate, sodium pyruvate, and nonessential amino acids (incomplete medium), and resuspended in complete medium (incomplete minimal essential medium plus 10% FCS and 2-mercaptoethanol) (20, 21). In these experiments, it was first necessary to incubate spleen cells with phenol/water–extracted LPS, 50 μg/5 × 10⁶ cells/ml for 18 h. The B cell population was then prepared by treatment of the nonadherent cells with anti-T cell mAb cocktail, followed by a Ficoll-Hypaque gradient separation. These LPS-pretreated B cells were added to macrol culture plates that contained adherent accessory cells. For preparation of adherent cells, spleen cells were treated with the anti-T cell cocktail, followed by 3,000 rad irradiation (Gamma Cell 1000; Atomic Energy of Canada Ltd., Kanata, Ontario, Canada). Cells were added to macrol culture plates, and, after 2 h incubation at 37°C, nonadherent cells were removed by aspiration, and individual wells were washed three to five times to completely remove nonadherent cells. Th cell-enriched (Lyt-1⁺) fractions were prepared by passing PP cells from SRBC-primed mice through nylon wool columns (20). The eluted cells were then treated with anti-mouse Ig serum (Meloy Laboratories Inc., Springfield, VA), and anti-Lyt-2 as previously described (20, 22).

Purification of IBFα. To purify IBF from T-T hybridoma supernatants and determine their isotype specificity, Sepharose 4-B columns, conjugated with purified monoclonal IgM (H5-3), IgG1 (H35-3), or IgA (MOPC 315) were used. Hybridoma supernatants (35–40 ml) from cells grown in FCS-free, HB 102 media were passed over these columns, and the effluent collected and concentrated by negative-pressure dialysis to the original volume. IBF fractions were obtained by elution with glycine-HCl (pH 2.8) buffer into an equal volume of borate-saline (pH 8.2) buffer (eluate). The eluate was concentrated to 1–3 ml by negative-pressure dialysis against RPMI 1640. The effluent and eluate were tested for their ability to enhance or suppress IgA responses in appropriate cultures containing antigen, as described below.

In Vitro Immune Responses. Purified B cells from normal mice, and appropriate aliquots
of culture supernatants or column-purified IBFα from T-T hybridomas were added to macroculture plates (Linbro Chemical Co., Hamden, CT) and immunized with SRBC, horse red blood cells (HRBC), or TNP-SRBC. Cultures were incubated at 37°C in an atmosphere of 7% O₂, 10% CO₂, and 83% N₂ (19-22). To examine the regulatory effects of IBFα on IgA responses, PP cell cultures (2.5 × 10⁶) from SRBC-primed mice were immunized with SRBC in the presence of different concentrations of column-purified IBFα. In addition, these IBFα were tested with cultures containing purified Lyt-1⁺ T cells (10⁶ cells/culture) from PP of orally SRBC-primed mice, and normal splenic B cells (2.5 × 10⁶ cells/culture) in the presence of SRBC.

**PFC Assay.** After 5 d of culture, nonadherent cells were removed from macroculture wells by vigorous pipetting, and washed in Hanks' balanced salt solution (Gibco Laboratories) and resuspended in Hank's at appropriate dilutions for PFC assay. Cultures were assessed for direct (IgM) and indirect (IgG and IgA) anti-SRBC, -HRBC, or -TNP PFC responses as previously described (19, 21).

**Measurement of Murine IgM, IgG, and IgA by Radioimmunoassay.** Synthesis of IgM, IgG, or IgA in splenic or PP B cell cultures treated with various hybridoma supernatants was measured after 5 or 7 d of culture, as previously described (31, 32). Briefly, polystyrene wells (Micro-ELISA, Dynatech Laboratories, Inc., Alexandria, VA) were coated with IgG fractions of either rabbit anti-mouse µ, γ, or α diluted in PBS, pH 7.2. Wells were aspirated, and protein-binding sites were blocked with 1% bovine serum albumin (radioimmunoassay grade, Sigma Chemical Co.) in PBS. Standards of IgM (MOPC 104E), IgG (affinity-purified mouse serum IgG) (Cappel Laboratories, Cochranville, PA), and IgA (MOPC 315), over a range of 0.25–25 ng/well, and dilutions of test supernatants were incubated in coated wells for 16 h at 25°C. After washing three times with PBS, ¹²⁵I-labeled anti-µ, -γ, or -α was added to appropriate wells, and incubated overnight. The unbound radioactivity was removed by extensive washing with PBS, and individual wells were counted in a gamma counter (Beckman Instruments, Inc., Palo Alto, CA). The IgM, IgG, and IgA levels present in cell culture supernatants were determined by reference to standard curves generated for each experiment.

**Statistics.** Values for PFC assay are expressed as the mean PFC response per culture ±SEM. Rosette formation is expressed as the range per triplicate determinations. The significances of differences between means of PFC responses were determined by the student's t-test.

**Results**

**Characteristics of T-T Hybridomas and Supernatants Derived From PP Th A Clones.** PP Th A clones 1 and 9 were separately fused with R1.1 T lymphoma cells, or with hybridomas produced in selective medium. Cells from each culture well showing hybridoma growth were tested for their ability to support in vitro IgA responses. Positive cultures were subcloned by limiting dilution. A total of 85 cloned cell lines were derived for subsequent characterization. Of this total, 66 hybridomas were obtained from the PP Th A clone 1 fusion, and 19 hybridoma lines were established from PP Th A clone 9. These cell lines have been designated Th HA to indicate that they are hybridomas derived from T helper cell clones that promote IgA responses. Culture supernatants from each of the 85 cell lines were tested for their ability to support IgA responses against SRBC in splenic B cell cultures from C3H/HeN (H-2k) mice. In preliminary studies, we found that B cell cultures triggered with LPS (see Materials and Methods) were required to detect IgA responses with some culture supernatants.

Of the 85 hybridoma supernatants tested, 16 supported IgA anti-SRBC PFC responses (Table I). 13 of these were derived from PP Th A 1, and three were from fusions of PP Th A clone 9 (Table I). Our previous studies (21, 22) had
Table I

Supernatants from FcaR⁺ Th HA Support In Vitro IgA Responses in LPS-driven B Cell Cultures

| Supernatant source | Anti-SRBC PFC per culture* | | |
|-------------------|---------------------------|---|---|
|                   | IgM | IgG | IgA |
| FcaR⁺             |     |     |     |
| Th HA₁            |     |     |     |
| 1                 | 339 ± 43 | 0 | 345 ± 32 |
| 2                 | 215 ± 16 | 79 ± 19 | 548 ± 43 |
| 3                 | 220 ± 24 | 115 ± 10 | 460 ± 52 |
| 4                 | 273 ± 30 | 0 | 534 ± 47 |
| 5                 | 393 ± 21 | 0 | 466 ± 23 |
| 6                 | 323 ± 52 | 52 ± 9 | 382 ± 36 |
| 7                 | 224 ± 61 | 35 ± 4 | 538 ± 62 |
| 8                 | 428 ± 39 | 73 ± 15 | 562 ± 35 |
| 9                 | 234 ± 34 | 116 ± 11 | 514 ± 44 |
| 10                | 251 ± 17 | 56 ± 9 | 562 ± 40 |
| 11                | 319 ± 43 | 0 | 557 ± 59 |
| 12                | 434 ± 28 | 0 | 347 ± 66 |
| 13                | 399 ± 41 | 0 | 390 ± 52 |
| Th HA₂            |     |     |     |
| 14                | 225 ± 20 | 0 | 515 ± 23 |
| FcaR⁻             |     |     |     |
| Th HA₁            |     |     |     |
| 30                | 328 ± 41 | 0 | 11 ± 3 |
| 37                | 245 ± 19 | 2 ± 2 | 6 ± 3 |
| 42                | 308 ± 21 | 5 ± 1 | 12 ± 5 |
| 51                | 279 ± 37 | 0 | 7 ± 2 |
| Th HA₂            |     |     |     |
| 70                | 288 ± 22 | 0 | 9 ± 4 |
| R1.1              |     |     |     |
| None              | 147 ± 31 | 2 ± 1 | 3 ± 1 |

Spleen cells were cultured with LPS (phenol/water–extracted) (5 × 10⁶ cells/50 μg LPS/ml) for 18 h. Nonadherent cells were then treated with anti-T cell mAb cocktail and rabbit complement, and cultured (2.5 × 10⁶ cells/well) with SRBC (1–2 × 10⁵) in the presence of adherent cells (macrophages) and supernatants from Th HA. PFC responses were assessed on day 5 of culture.

* Values are the mean anti-SRBC PFC per culture from triplicate culture for each experiment, for three separate experiments.

† Subscript refers to PP Th A parent cell origin.

Shown that PP Th A clone 1 supports some IgM responses, and elevated IgA responses, while clone 9 supports low IgM, IgG1, IgG2, and IgG3 responses, and high IgA responses. A total of 7 of 13 hybridomas from clone 1 supported some IgG responses, and one of three hybridoma supernatants from clone 9 gave IgG responses (Table I). However, the R1.1 supernatant did not support the IgG isotype in LPS-triggered cultures. Although the reason for these IgG responses remains unclear, it may be due to the LPS triggering event, since it is
well known that LPS induces polyclonal responses among the IgG subclasses. This possibility is further supported by lack of IgG PFC responses in normal PP B cell cultures treated with Th HA supernatants (see below). In these studies, SRBC immunization of LPS-triggered splenic B cells, in the absence of hybridoma supernatants, resulted mainly in IgM anti-SRBC PFC responses (Table I). Culture supernatants from non-IgA-supporting hybridomas did not induce either IgG or IgA isotype responses, although these fractions generally enhanced the in vitro IgM PFC response (Table I). Both of the original clones (PP Th A 1 and 9) were Thy-1.2+ and Lyt-1+,2− and did not express I-Ak (21). All 16 hybridomas which produced soluble factors that supported IgA responses expressed the Thy-1.2 antigen but were Lyt-1+,2−. 66 out of 69 of the remaining hybridomas also exhibited this pattern of surface antigen expression, while 3 of 69 were Thy-1.2+, Lyt-1+,2−.

All 16 hybridomas whose supernatants support IgA responses to SRBC bear Fc receptors for IgA (FcaR+) (Table II). On the other hand, none of the 69 hybridoma lines whose supernatants failed to support IgA responses expressed FcaR. No immunocytoadherence was seen when the 16 hybridomas were treated with either IgM or IgG anti-TNP mAb followed by incubation with TNP-

### Table II

| FcR isotype-specificity (rosette formation) | FcaR |
|-------------------------------------------|------|
| %                                         |      |
| Th HA1,2                                 |      |
| 1                                         | 53-76|
| 2                                         | 68-81|
| 3                                         | 59-68|
| 4                                         | 34-47|
| 5                                         | 51-70|
| 6                                         | 66-74|
| 7                                         | 55-62|
| 8                                         | 28-41|
| 9                                         | 30-45|
| 10                                        | 56-71|
| 11                                        | 82-94|
| 12                                        | 60-72|
| 13                                        | 51-66|

| Th HA3                                  |      |
| 14                                       | 85-94|
| 15                                       | 36-46|
| 16                                       | 30-44|

Th HA hybridomas were reacted with either IgM (H5-3), IgG1 (H35-3), or IgA (MOPC 315) anti-DNP mAb for 1 h at 37°C. Hybridoma cells binding more than five red cells were counted as positive. Rosettes were counted in 10-15 fields. >1,000 Th HA cells were scored on each slide for each experiment.

* Rosette formation is expressed as percentage and the range of triplicate determinations are presented. No FcαR- or FcγR-bearing hybridomas were found.

1 Subscript refers to PP Th A parent cell origin.
conjugated SRBC, indicating that these hybridomas express neither Fc_\gamma R or Fc_\alpha R (Table II). To verify this, each hybridoma cell line was incubated with purified MOPC 315 IgA, washed, and stained with rabbit F(ab')_2 FITC-labeled anti-mouse \( \alpha \), and subjected to flow cytometry. All 16 hybridomas gave strong positive staining, confirming the presence of Fc_\alpha R on their surface. None of the hybridomas exhibited immunofluorescence when treated with purified IgM or IgG1 and FITC-F(ab')_2 anti-heavy chain, and analyzed on a FACS.

**B Cell and Antigen Requirements for Th HA Hybridoma Supernatant Support of IgA Responses.** Since PP Th A clones are SRBC-specific and require antigen for in vitro IgA responses, we next determined whether Th HA hybridomas produce an antigen-specific, IgA-promoting factor, or whether the supernatants all support antigen-induced responses. As summarized in Table III, supernatants from eight Th HA_1 hybridomas, and Th HA_9-14 all support SRBC, HRBC, or TNP-specific IgA PFC responses in LPS-triggered splenic B cell cultures. The responses were specific for the antigen used in culture, no polyclonal PFC were seen. No significant IgA PFC responses occurred in B cell cultures containing Th HA supernatants in the absence of antigen (Table III). This shows that IgA PFC responses require T-dependent antigen for Th HA supernatant support of IgA responses.

Murine PP contain a significant population of sIgA⁺ cells, and enrichment of this cell type results in higher IgA responses in the presence of PP Th A cells (22). When soluble Th HA factors were added to PP B cell cultures and immunized with T-dependent antigens, good IgA PFC responses to SRBC,

**Table III**

| Supernatant source | Antigen stimulating IgA response (PFC per culture) | SRBC | HRBC | TNP-SRBC |
|--------------------|---------------------------------------------------|------|------|----------|
| Th HA_1            |                                                   |      |      |          |
| 1                  | 359 ± 40 (10 ± 4)                                  | 265 ± 39 (7 ± 4) | 329 ± 36 (7 ± 3) |
| 3                  | 488 ± 49 (11 ± 3)                                  | 367 ± 48 (5 ± 3) | 409 ± 48 (5 ± 1) |
| 4                  | 540 ± 55 (7 ± 2)                                  | 398 ± 57 (6 ± 2) | 512 ± 83 (7 ± 3) |
| 5                  | 490 ± 61 (10 ± 3)                                  | 332 ± 51 (8 ± 4) | 539 ± 41 (4 ± 2) |
| 7                  | 545 ± 37 (5 ± 1)                                  | 401 ± 30 (7 ± 3) | 498 ± 31 (10 ± 2) |
| 8                  | 604 ± 91 (6 ± 2)                                  | 425 ± 72 (10 ± 4) | 512 ± 58 (8 ± 3) |
| 9                  | 525 ± 36 (8 ± 4)                                  | 371 ± 19 (7 ± 2) | 480 ± 39 (7 ± 2) |
| 10                 | 582 ± 39 (5 ± 2)                                  | 329 ± 28 (5 ± 2) | 373 ± 52 (6 ± 3) |
| Th HA_9-14         |                                                   | 409 ± 45 (4 ± 1) | 432 ± 21 (6 ± 2) |
| R1.1               | 2 ± 2 (0)                                         | 1 ± 1 (0)       | 2 ± 2 (0)       |

See Table I for culture details. Briefly, LPS-triggered B cells (2.5 × 10⁶ cells/well) were incubated with SRBC, HRBC, or TNP-SRBC (or without antigen) in the presence of Th HA supernatants, and IgA PFC were assayed on day 5 of culture.

* Values are the mean IgA PFC per culture ±SEM from triplicate cultures for each experiment, and four separate experiments. Numbers in parenthesis are IgA PFC per culture, without antigen. IgA PFC responses without Th HA supernatants were: SRBC (0–3 cells/culture); HRBC (0–2 cells/culture), and TNP-SRBC (0–4 cells/culture). No polyclonal PFC were induced with specific antigen and Th HA supernatants.
HRBC, and TNP were seen (Table IV). However, identically treated splenic B

TABLE IV

Supernatants from Th HA Support In Vitro IgA Responses in Normal PP B Cell Cultures

| Supernatant source | Antigen stimulating IgA response (PFC per culture)* | SRBC | HRBC | TNP-SRBC |
|--------------------|-----------------------------------------------------|------|------|----------|
| Th HA<sub>1</sub>   |                                                     |      |      |          |
| 1                  | 381 ± 43 (8 ± 4)                                    | 206 ± 29 (7 ± 3) | 366 ± 19 (6 ± 1) |
| 3                  | 520 ± 86 (8 ± 3)                                    | 348 ± 45 (6 ± 2) | 497 ± 51 (6 ± 2) |
| 4                  | 554 ± 69 (4 ± 2)                                    | 356 ± 38 (5 ± 4) | 501 ± 23 (9 ± 3) |
| 5                  | 483 ± 47 (9 ± 5)                                    | 312 ± 44 (7 ± 3) | 514 ± 52 (8 ± 2) |
| 7                  | 549 ± 31 (4 ± 3)                                    | 409 ± 42 (6 ± 3) | 486 ± 11 (4 ± 1) |
| 8                  | 492 ± 55 (6 ± 3)                                    | 354 ± 42 (7 ± 1) | 491 ± 43 (5 ± 2) |
| 9                  | 503 ± 26 (7 ± 2)                                    | 328 ± 26 (5 ± 2) | 398 ± 40 (6 ± 1) |
| 10                 | 547 ± 49 (5 ± 1)                                    | 311 ± 27 (4 ± 2) | 409 ± 28 (5 ± 1) |
| Th HA<sub>9</sub>   |                                                     |      |      |          |
| 14                 | 488 ± 37 (6 ± 2)                                    | 350 ± 26 (3 ± 2) | 421 ± 36 (4 ± 2) |

R1.1                | 1 ± 1 (0)                                           | 0 (0) | 2 ± 1 (0) |

PP cells were treated with a cocktail of anti-Thy-1.2, anti-Lyt-1, and anti-Lyt-2 mAb, plus rabbit complement. PP B cells (2.5 × 10<sup>6</sup>) were cultured with SRBC, HRBC, or TNP-SRBC (or without antigen) in the presence of supernatants from Th HA. IgA anti-SRBC, HRBC, or TNP PFC responses were assessed on day 5 of culture.

Values are the mean IgA PFC per culture ±SEM from triplicate cultures for each experiment, for three separate experiments. Numbers in parenthesis are IgA PFC per culture without antigen. IgA PFC responses without Th HA soluble factors were: SRBC (2-9 cells/culture), HRBC (2-5 cells/culture), and TNP (3-9 cells/culture). IgG PFC responses with Th HA supernatants were: SRBC (0-5 cells/culture), HRBC (0-3 cells/culture), and TNP (0-8 cells/culture).

TABLE V

Supernatants from Th HA Do Not Support Polyclonal Ig Responses

| Supernatant source | Source of B cell cultures | Total Ig synthesis* | IgM | IgG | IgA |
|--------------------|----------------------------|--------------------|-----|-----|-----|
| Th HA<sub>1</sub>  | PP                         | ng/ml              | 675 | 412 | 389 |
| 5                  |                            |                    | 702 | 581 | 411 |
| 9                  |                            |                    | 798 | 300 | 432 |
| Th HA<sub>9</sub>  |                            |                    | 813 | 496 | 443 |
| 14                 | Spleen                     |                    | 30,022 | 879 | 413 |
| 9                  |                            |                    | 28,435 | 904 | 450 |
| Th HA<sub>9</sub>  |                            |                    | 25,751 | 1,038 | 404 |
| 14                 |                            |                    | 31,476 | 955 | 431 |

PP or LPS-pretreated splenic B cell cultures (see Tables I and IV legends) were incubated with supernatants from Th HA for 5 d.

* Culture supernatants were harvested, and total IgM, IgG, or IgA syntheses were measured by radioimmunoassay (see Materials and Methods).
cell cultures (non-LPS-stimulated) gave essentially no IgA responses (data not shown). No IgG PFC responses were noted in immunized PP B cell cultures in the presence of any of the 16 Th HA supernatants. These results suggest that Th HA supernatants contain factor(s) that allow selective triggering of committed slgA+ B cells for this isotype response.

It could be argued that Th HA supernatants contain factors which induce polyclonal IgA responses, and that this would not be detected with IgA PFC response assays to heterologous erythrocytes or to hapten. Therefore, in other experiments, soluble Th HA factors were added to either normal PP B cells, or LPS-triggered splenic B cells, and IgM, IgG, and IgA synthesis was measured in culture supernatants. As can be seen in Table V, these supernatants did not enhance Ig synthesis in PP B cell cultures over the base line controls (no soluble factors added). With splenic B cells, prior treatment with LPS resulted in significant IgM and some IgG synthesis within 5 d, however, no enhancement of IgM or IgG synthesis occurred in the presence of Th HA supernatants. IgA synthesis was minimal in all cultures tested (Table V). When cultures were incubated for 7 d and Ig measured in the culture supernatants, the pattern of Ig synthesis was unchanged (data not shown). We conclude from these experiments that Th HA soluble factors do not possess polyclonal B cell activation properties, nor do they support polyclonal IgA synthesis.

**Th HA Supernatants Contain IBFα That Regulate Antigen-dependent IgA Responses.** Recent studies (16, 17) have shown that T cell hybridomas that possess FcaR can be induced to release IBFα, presumably the released form of FcaR. We have considered the possibility that the 16 Th HA cell lines synthesize and secrete IBFα, which could account for the promotion of IgA responses by these supernatants. To test this, aliquots of three separate supernatants were passed over immunoadsorbent columns (IgA, IgM, or IgG), and the effluents (non-Ig-bound) and eluates were tested separately for promotion of IgA responses. Passage over the IgA column removed the factor(s) which promoted IgA PFC responses from all three supernatants tested. The factor(s) could be recovered in the eluate, as shown by its ability to support good IgA responses (Fig. 1). In contrast, passage of supernates over IgM or IgG columns failed to remove the active fractions, clearly indicating that the factor(s) does not bind to these Ig. These results indicate that promotion of IgA responses by Th HA supernatants is largely due to the presence of IBFα. In another experiment, we have shown that the eluate from a MOPC 315 IgA column, when passed again over this immunoadsorbent, left no IgA-promoting activity in the effluent. However, full activity was recovered in the eluate, and preincubation of the eluate with purified IgA, but not F(ab')2 of IgA, IgM, or IgG, completely abrogates IgA PFC responses (data not shown).

Purified eluates from four different Th HA supernatants were tested over a wide concentration range for enhancement of IgA responses in SRBC-immunized PP B cell cultures. As can be seen in Fig. 2, higher concentrations of IBFα resulted in lower IgA responses, dilutions of ~1:10 gave highest IgA responses. Three of the four IBFα gave a similar dose-response pattern, while IBFα from Th HA-10 exhibited somewhat lower IgA-enhancement activity. Column eluates from R1.1 supernatants (Fig. 2), or from non-IgA-enhancing culture
FIGURE 1. Detection of IBF present in Th HA supernatants by affinity chromatography. Aliquots (35-40 ml) of hybridoma supernatants from Th HA-9 ( ), -10 ( ), or -14 ( ) grown in FCS-free HB 102 media were passed over IgA, IgM, or IgG Sepharose 4-B immunoadsorbent columns. The effluents and eluates (see Materials and Methods) were tested separately for their ability to support IgA anti-SRBC PFC responses in PF B cell cultures. Control cultures (PP B cells with column fractions and no antigen) gave responses ranging from 4 to 17 IgA PFC per culture. Cultures with SRBC and no column fraction yielded 3-9 IgA PFC per culture.

fractions (data not shown) exhibited no IgA-promoting activity in SRBC-immunized B cell cultures. These results suggested that optimal concentrations of IBFa are required for in vitro IgA responses, and that higher concentrations are suppressive.

To determine whether IBFa regulates T-dependent responses, PP cell cultures from orally SRBC-primed mice were cultured in vitro with SRBC, a procedure previously shown to yield good IgA responses (19), and the effect of purified IBFa was assessed. Higher concentrations of IBFa were suppressive (Fig. 3A), while less IBFa enhanced in vitro IgA anti-SRBC PFC responses. Similar results were also noted when these concentrations of IBFa were tested in splenic B cell cultures containing PP Th from orally primed mice (Fig. 3B). The pattern of IBFa regulation was similar in both culture systems, and the two highest IBFa concentrations suppressed, while the next three lower doses enhanced IgA responses.

Discussion

Studies with cloned T cells from murine PP or human T cell hybridomas have suggested at least two major mechanisms for T cell regulation of IgA responses. Kawanishi et al. (23, 24) have produced PP T cell clones which induce slgM+ B
cells to switch to expression of sIgA. The post-switched sIgA+ B cells were only induced to secrete IgA in the presence of B cell growth and differentiation factors (33). This suggested that switch T cells are major determinants for B cell commitment to IgA, and that isotype-specific regulatory T cells are not required for IgA synthesis. Contrary to this, our studies with antigen-induced, IgA isotype-specific clones of PP Th A cells indicate that GALT Th cells select already committed (sIgA+) cells from the B cell subpopulation for IgA responses (21, 22). These PP Th A cells bear FcaR, and may recognize the Fco− determinant on committed, sIgA+ B cells, thus inducing B cell differentiation to IgA synthesis through either direct cell contact, or via secreted FcaR. In support of this is the finding that preincubation of PP Th A cells with purified IgA, but not F(ab')2 of IgA (or of the IgM or IgG subclasses), abrogates IgA-specific responses (30). The FcoR may not represent the only determinant involved in isotype regulation, since elegant studies by Mayer and coworkers (34) have shown that human T cell hybridomas express mainly FcμR, but secrete factor(s) that support IgA responses. It is interesting that maximum IgA responses occurred with B cells already committed to the IgA isotype, and implied that non–IgA-binding factors or α-lymphokines may also regulate IgA responses.

To determine the molecular basis for IgA response regulation, we have produced T-T hybridomas (Th HA cells) from two well-characterized PP Th A clones (1 and 9). Th HA culture supernatants did not induce IgA responses in normal splenic B cell cultures immunized with T-dependent antigens, however, prior stimulation of B cells with LPS (18 h) followed by immunization with T-dependent antigen in the presence of Th HA soluble factors resulted in IgA responses. Certain Th HA supernatants also supported small, antigen-dependent IgG responses (Table I). The reason for the IgG response is not yet clear,
LG A-BINDING FACTOR REGULATION OF LG A RESPONSES

A. PP Cell Cultures

Dilution Of Purified IBFa Employed

FIGURE 3. IBFa regulation of T-dependent IgA anti-SRBC PFC responses. Dilutions of purified IBFa from Th HA-9 (○) or -10 (□) were assessed for suppression or enhancement of IgA responses in either (A) whole PP cell cultures from orally SRBC-primed mice or (B) splenic B cell cultures containing PP Th cells and SRBC. The horizontal dashed line indicates the level of IgA anti-SRBC response in the absence of IBFa.

however, it is known that LPS triggers IgM and certain IgG-subclass polyclonal responses (35). It is possible that LPS triggers clones of IgG subclasses, and, together with T-dependent antigen, would result in specific IgG PFC responses. This explanation was borne out by our finding that Th HA supernatants did not promote significant IgG responses in normal PP B cell cultures, although IgA responses were clearly enhanced (Table IV). Thus, we have no evidence that Th HA supernatants contain IgG-enhancing factors, although additional studies will be required to firmly establish this point.

Each Th HA cell line which secreted factor(s) that supported in vitro IgA responses to T-dependent antigen was shown to express FcaR (Table II). This finding raised the possibility that released FcaR, or IBFa, may account for the ability of Th HA culture supernatants to support IgA responses. This suggestion was verified by experiments which showed that the factors which promoted IgA responses were selectively removed by IgA (but not by IgM or IgG) affinity columns (Fig. 1). Furthermore, elution of the IBFa from the column allowed
recovery of biologically active components, which, when added to PP B cell cultures, supported IgA-isotype responses. Interestingly, the most concentrated IBFα supported less IgA response, while diluted (1:10) samples gave maximum support (Fig. 2). It is possible that excessive amounts of IBFα may bind to slgA+ B cells and actually inhibit isotype responses.

Recently, others (16, 17) have characterized released FcaR from a T cell hybridoma line, T2D4, which simultaneously expresses both FcγR and FcaR. When T2D4 cells were incubated at 37°C with purified mouse IgA in medium containing FCS, the FcaR was selectively released (16). In other studies, they showed that Con A-stimulated spleen cells, when incubated with IgA, released IBFα into the culture supernatant (17). Purification of these IBFα by IgA affinity chromatography yielded molecules capable of suppressing pokeweed mitogen-driven polyclonal IgA responses (16, 17). When FcγR+ T cells were removed from Con A-stimulated cultures, production of IBFα was eliminated, suggesting that IBFα-releasing cells are also FcγR+ (17). This is consistent with the simultaneous expression of FcγR and FcaR on the T2D4 T cell line, and suggests that T cells first express FcγR before they express FcaR (17). In this regard, it is possible that T cells may first express either FcμR or FcγR before induction (by IgA) of the FcaR. One might speculate that the hybridomas described by Mayer et al. (34) express mainly FcμR, but that they may produce factors which bind IgA, and support IgA responses in cultures containing cells committed to this isotype.

Others (9-12, 36-41) have extensively characterized potentiating and suppressing IBFε produced by rat FcεR+ T cells that acted on slgE+ committed B cells to regulate isotype-specific immune responses. The IgE-potentiating factor binds IgE, is ~15,000 mol wt, and possesses a mannose-rich oligosaccharide responsible for binding to lentil lectin and Con A (9, 10, 12, 36-38, 40, 41). The IgE-suppressive factor is of similar molecular weight, also binds IgE, but lacks the mannose-rich polysaccharide moiety (9, 11, 12, 37-39, 41). Recent studies (42, 43) in mice suggest that similar IBFε regulate murine IgE responses.

The IBFα produced by Th HA cell lines reported here support antigen-dependent IgA responses, in contrast to the IBFα (16, 17) and IBFε (9-12, 36-43) described above, which regulate the global isotype-specific response. Thus, both T-dependent antigen and IBFα are required for IgA responses. However, the addition of IBFα to cultured B cells, alone or with antigen, did not induce polyclonal IgA synthesis (Table V). Furthermore, an optimal amount of IBFα was required for promotion of IgA responses; higher concentrations were suppressive (Fig. 2), a point considered in more detail below. Thus, a dual triggering event is required, i.e., binding of antigen to specific clones of slgA+ B cells, and a second signal, provided by binding IBFα to the Fc of membrane IgA. When both signals are produced, the committed B cell is activated, and induced to differentiate into cells that synthesize specific IgA. Th HA supernatants may also contain B cell differentiation factors. However, it is clear that the IgA-promoting factors bind IgA, and it would be necessary to postulate the existence of a B cell differentiation factor specific for IgA. If it can be shown that IBFε binds to the Fc of membrane IgA, and (in the presence of antigen) triggers final differentiation to plasma cells synthesizing IgA, then it would be appropriate to classify
these IBFα molecules as IgA-specific B cell differentiation factors. In this regard, we are currently characterizing these IBFα for molecular size, heterogeneity, and protein and glycoprotein content, the results of which will be the subject of a future paper.

To date, IBFα has been obtained that exhibits two apparently different functions. Our studies show that IBFα promotes specific IgA responses to various T-dependent antigens, and does not induce polyclonal IgA responses. Higher concentrations of IBFα suppress in vitro IgA responses, while lesser amounts enhance this isotype response (Fig. 3). Other studies (16, 17), described above, suggest that released FcαR, or IBFα, can suppress polyclonal IgA responses. Two explanations may be given for these apparent differences. The first is that only one class of IBFα is produced, and depending upon its concentration and the experimental conditions employed for bioassay, it could either promote or suppress IgA responses. Thus, in our assays, LPS-triggered splenic B cells or normal PP B cell cultures (with high frequencies of sIgA⁺ B cells), help, or, enhancement by IBFα is seen. Furthermore, higher concentrations of IBFα, in cultures containing isotype-specific Th cells and IgA B cell precursors, down-regulate the IgA response. This may explain the results of others (16, 17), which show that released FcαR suppressed pokeweed mitogen–driven polyclonal IgA responses. The second possibility is that, like the IBFε-regulated IgE system, two classes of IBFα are produced, one of which promotes, and the second of which suppresses IgA responses. Our Th HA cells may produce less suppressive factor(s), thus, a high concentration was required for the observed effect. The major product of these cell lines would be a helper form of IBFα that enhances antigen-dependent IgA responses. In the other studies described above (16, 17), the T2D4 hybridoma, or Con A-stimulated T cells may produce mainly suppressive forms of IBFα. We are currently purifying the IBFα produced by different Th HA cell lines to homogeneity, to help resolve this important issue.

Summary

T–T hybridomas, produced by fusions between R1.1 T lymphoma and cloned T helper cells that promote IgA responses (Th A cells) were characterized in this study. A total of 85 cloned cell lines were produced, and their supernatants were assessed for support of antigen-dependent IgA (and IgM and IgG) responses. 16 of 85 culture fractions supported IgA anti–sheep red blood cell, –horse red blood cell, or –trinitrophenyl responses in either lipopolysaccharide-triggered splenic B cell, or normal Peyer’s patch B cell cultures, and the responses were specific for the antigen used for in vitro immunization. None of the supernatants from the cell lines induced significant polyclonal responses in these B cell cultures. Interestingly, the 16 hybridomas that produced supernatants with IgA-promoting properties had Fc receptors for IgA (FcαR), but did not express FcεR or FcγR. When supernatants from FcαR⁺ T cell lines were subjected to IgA affinity chromatography, the IgA-promoting activity bound to IgA (IBFα) and was recovered in the eluate. No binding of active fractions occurred when supernates were passed through IgM or IgG immunoadsorbent columns. High concentrations of purified IBFα suppressed T-dependent IgA responses, while an optimal level was required for enhancement of this isotype response. These results suggest
that FcαR⁺ hybridomas derived from Th A cells release IBFcα into the culture medium, and that these molecules regulate IgA responses to various T-dependent antigens.

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