B CELL HELPER FACTORS

I. Requirement for Both Interleukin 2 and Another 40,000 Mol Wt Factor*

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Supernatants (Sn)¹ from T cell-containing cultures stimulated with either antigens or mitogens have been the source of nonspecific helper factors for many years (1–6). Such Sn have the ability to restore the antibody response of T cell-depleted spleen cells to the antigen, sheep erythrocytes (SRBC). Some of the active principle(s) in these Sn have been shown to have an ~30,000–40,000 mol wt by Sephadex chromatography (2–6). Recently, one factor shown to be present in this fraction is interleukin 2 (IL-2; formerly, T cell growth factor) (7–8). Although purified IL-2 has been shown to stimulate B cell responses to SRBC, its powerful T cell growth-promoting activity has left open the possibility that its activity in B cell responses is mediated indirectly by its stimulation of the small number of T cells contaminating most B cell preparations and that other factors are the ultimate stimulators of B cell responses.

We continued our comparison of the helper T cell-replacing activity of IL-2 to that of other factors present in the Sn of normal murine spleen cells stimulated with Concanavalin A (normal Con ASn) (9). In the in vitro response of B cells to SRBC, both a factor indistinguishable from IL-2 and a second factor(s) with an ~40,000 mol wt were found to be essential in replacing helper T cell activity when using severely T cell-depleted B cells, although IL-2 alone was sufficient in the presence of only a few contaminating T cells. Our results suggest that IL-2 can influence antibody responses through both an obligatory direct effect on responding B cells and an indirect effect via the stimulation of T cells that produce a second required factor(s).

Materials and Methods

Mice. B6D2F1, B6AF1, C57BL/10SgSn (B10), B10.A, B10.D2, B10.BR AKR, and C58 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, or in some cases were bred in our facilities from breeding stock obtained from The Jackson Laboratory. The following mice were bred in our facilities from breeding stock obtained from the sources indicated: D1.LP, B10.A(5R), and B10.M (The Jackson Laboratory); B10.A(4R) (Dr. J. Stimpfling, ‡ Work done during the tenure of an established investigatoryship from the American Heart Association and with funds contributed in part by the Colorado Heart Association.

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Abbreviations used in this paper: α-MM, α-methyl mannoside; ATS, anti-thymocyte serum; B10, C57BL/10SgSn; C, complement; Con A, concanavalin A; IL-1, interleukin 1; IL-2, interleukin 2; PFC, plaque-forming cells; Sn, supernatant(s); SRBC, sheep erythrocytes; TRF, T cell replacing factor.

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Rocky Mountain National Laboratory, Great Falls, Mont.; B10.AQR (Dr. C. David, The Mayo Clinic, Rochester, Minn.); B10.S(7R), A.TH, A.TL, and A.AL (Dr. D. Shreffler, Washington University, St. Louis, Mo.). Outbred nu/nu mice were bred in our own facilities from nu/+ stock.

Antigens and Other Reagents. SRBC from a single animal (220) were obtained from Colorado Serum Company, Denver, Co. Con A was purchased from Miles-Yeda, Rehovoth, Israel, and α-methylmannoside (α-MM) was obtained from the Sigma Corporation, St. Louis, Mo.

Cultures. Cell suspensions were cultured by modifications (10, 11) of the methods of Mishell and Dutton (12). B cell responses to SRBC were obtained in 96-well microculture plates (Costar Data Packaging, Cambridge, Mass.). Unless stated otherwise, cultures contained 1.5 × 10⁵ T-cell depleted spleen cells in a volume of 0.1 ml, SRBC at a concentration of 0.05%, and α-MM at a concentration of 10 mg/ml. Anti-SRBC plaque-forming cells (PFC) were enumerated on day 4 of culture, using an in situ PFC assay (10). Results are reported as the total number of anti-SRBC PFC assayed in twelve identical microculture wells.

T Cell Hybridomas. We originally described the T cell hybrid FS6-14.13, which was derived from the fusion of the AKR thymoma, BW5147, to normal T cells from B6D2F1 mice. This hybrid was demonstrated to produce IL-2 when stimulated with Con A (9). In most experiments described below this hybrid was used as the source of IL-2.

In one experiment, the Con A-stimulated Sn of FS6-14.13 was compared to those produced with a series of similarly derived hybrids. FS7-8 and FS7-59 were derived from the fusion of BW5147 to normal T cells from B10.BR mice. FS8-1, FS8-4, FS8-5, FS8-7, FS8-14, and FS8-16 were derived from the fusion of BW5147 to normal T cells from AKR mice. These hybrids were chosen because when stimulated with Con A they produced a wide range of IL-2 concentrations from little or no IL-2 produced (e.g., FS8-5) to very high levels of IL-2 (e.g., FS7-59).

Con A Sn Production. Con A Sn was prepared from the T cell hybrids, as previously described, by incubation overnight at 5 × 10⁵ to 5 × 10⁶ cells/ml in medium containing 4 μg/ml Con A. The cell-free Sn was stored at −20°C until used.

To prepare normal Con A Sn B6AF1 or B6D2F1, spleen cells were cultured at 10⁷ cells/ml in medium containing 4 μg/ml Con A (3). The cell-free supernatant was then filtered through a 0.2 μm filter and stored at −20°C until used. In some experiments an IL-2-depleted normal Con A Sn was prepared by culturing spleen cells at 10⁵ cells/ml for 4 d with 4 μg/ml Con A. In this case, cultures were fed on day 1 and day 2 with Mishell/Dutton nutrient cocktail (12). The cell-free Sn was filtered and stored as above.

Concentration of Sn. A concentrated Con A Sn of FS6-14.13 was prepared by stimulating the hybrid cells for 24 h at 10⁶ cells/ml with 3 μg/ml Con A in medium containing only 0.5% fetal calf serum (FCS) (instead of the usual 5%). After the method of Watson et al. (5), α-MM was added to this Sn to a concentration of 0.1 M. Ammonium sulfate was then added to 40% of saturation, and the small precipitate was removed by centrifugation. Ammonium sulfate was then adjusted to 80% of saturation and the collected precipitate redissolved in a small volume of 0.15 M NaCl, dialyzed extensively against 0.15 M NaCl followed by balanced salt solution. This preparation was then sterilized by filtration and stored frozen until used. This procedure resulted in recovery of virtually all of the IL-2 in the original Sn, with an overall concentration of about 50-fold.

A 4-d normal Con A Sn depleted of IL-2 was concentrated similarly. Normal spleen cells were incubated for 4 d in medium containing 4 μg/ml Con A and 2.5% FCS. This Sn was fractionated with ammonium sulfate, dialyzed, and sterilized as above. Again the overall concentration was ~50-fold.

Assay for T Cell Growth-promoting Activity of IL-2. IL-2 preparations were assayed for their ability to stimulate T cell growth using the IL-2-dependent T cell line HT-2 (kindly given to us by Dr. J. Watson, University of California, Irvine, Calif.). Although originally reported as a cloned helper T cell line (13), the subline growing in our laboratory had lost its helper activity while retaining its absolute dependence on IL-2 for viability and growth. In the absence of IL-2, HT-2 was ≤5% viable after 24 h, whereas in the continuous presence of IL-2, viability was >95%. To assay for IL-2, 4 × 10⁵ viable HT-2 cells were cultured for 24 h in 0.1 ml of medium containing twofold dilutions of the unknown preparation. Cultures were then examined with an inverted phase microscope. Cultures containing >90% viable cells were scored positive.
Those with <10% viable cells were scored negative. Cultures with 10–90% viable cells were scored intermediate. For a given sample, the first dilution to be scored less than positive was defined as containing 1 U of IL-2. By this assay the 24-h normal Con A Sn and FS6-14.13 Con A Sn used in these experiments contained between 250–1,000 U of IL-2/ml.

Absorption of Con A Sn. Three cell preparations were used to absorb IL-2 from normal Con A Sn. The first was a preparation of normal spleen cells incubated at 10^7 cells/ml for 48 h with 4 μg/ml Con A (14). The second population was the nylon fiber nonadherent T cell blasts from these Con A-activated spleen cells. The third was the IL-2-dependent cell line, HT-2 (see above). Absorptions were performed by incubating normal Con A Sn for 20 h at 37°C with 1 × 10^7/ml Con A-activated spleen cells, 1 × 10^7/ml Con A-activated splenic T cells, or 5 × 10^6/ml HT-2 T cells. The cell-free supernatants were then filtered and stored at −20°C. As stated above, as a fourth method of producing IL-2-depleted normal Con A Sn, we cultured normal spleen cells at 10^7/ml in 4 μg/ml Con A for 4 d instead of 1 d. Such cultures first produce and then consume IL-2. Supernatants were then harvested, filtered, and stored at −20°C. All four methods of IL-2 depletion resulted in preparations that failed to support the growth of HT-2 cells even when used undiluted (<10 U of IL-2/ml).

In addition, similar absorptions were performed with various non-IL-2-dependent cells to establish the specificity of the IL-2 absorptions. These negative control absorptions were performed with normal spleen cells (10^7/ml), anti-T cell serum plus complement-treated 48-h LPS-induced B6D2F1 B cell blasts (10^7/ml), P3X63AG8.653, a nonimmunoglobulin-secreting variant of the BALB/c myeloma, MOPC-21 (5 × 10^6/ml), and BW5147 (5 × 10^6/ml). None of these absorptions removed IL-2 from the Con A Sn.

Antisera and Hybridoma Antibodies. Anti-thymocyte serum (ATS) was purchased from Microbiological Associates, Walkersville, Md. To prepare a T cell-specific reagent, ATS was absorbed as previously described (15) with the myeloma, XS63, obtained from the Cell Distribution Center, The Salk Institute, La Jolla, Calif. or the B cell tumor, CH-1, kindly provided by Dr. Noel Warner, University of New Mexico, Albuquerque, N. M. The rat anti-Thy-1 hybridoma T24/40.7 was obtained from Dr. Ian Trowbridge, The Salk Institute, La Jolla, Calif. Ascitic fluid from the anti-Qa-4 hybridoma, B16/146, and anti-Qa-5 hybridoma, B16/167, were kindly provided by Dr. Ulrich Hammerling, Sloan Kettering Memorial Cancer Institute, New York (16).

The hybridoma MK-2.2 was produced in this laboratory from the fusion of the BALB/c myeloma, 45.6TG1.7 (MPC-11) to spleen cells from A.TH mice that had been hyperimmunized with A.TL Con A-activated T cell blasts (17). The supernatants of the hybrids were screened with rabbit complement (C) for cytotoxic activity on normal A.TL T cells. MK-2 tested positive in this assay. The hybrid was cloned to yield MK-2.2, which was used in these studies.

Although in the original immunization we were attempting to produce anti-la antibodies, MK-2.2 turned out to have a very unusual auto-antibody specificity, as shown in Table I. When used with rabbit complement, MK-2.2 antibody killed >90% of splenic T cells in all strains tested regardless of background or H-2 type, including A.TH, the strain of origin. When tested with spleen cells that had been depleted of T cells with anti-Thy-1 antibody and C, MK-2.2 killed up to 30% of the cells. However, this non-T cell killing was linked to the D end of H-2. All strains that carried H-2D^d, including A.TH, showed ~20–30% killing of non-T cells. All other strains tested showed no or very little killing of non-T cells (0–10%). The B cells that survived treatment with MK-2.2 antibody and C in H-2D^d strains were unaffected in their in vivo response to a variety of antigens (TNP-Ficoll, TNP-lipopolysaccharide, and SRBC) (data not shown). MK-2.2, therefore, had an unusual reactivity reminiscent of a Qa specificity (16, 18, 19) in that it showed a linkage to H-2D and was detectable on T cells and non-T/non-B cells but apparently was absent from most B cells.

Depletion of T Cells from Spleen Cells. Spleen cells were thoroughly depleted of T cell activity as follows. 2 d before killing, mice were injected with 0.04 ml of ATS to remove recirculating T cells (20). Spleen cells from these mice were incubated at 0°C for 30 min at 3.3 × 10^7 cells/ml with an antibody cocktail containing the following ingredients: 5% T24/40.7 culture Sn; 1% B cell-absorbed ATS; 0.2% B16/146 and B16/167 ascitic fluid; and 4% of × 50 concentrated MK-2.2 culture Sn. The cells were then centrifuged, resuspended to the original
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Table I

Specificity of MK-2.2 Antibody

| H-2 regions | Tla |
|-------------|-----|
| K A B J E C S G D | T cells | Non-T cells |
| A.TH | s s s s s s s d a | >90% | 29 |
| B10.S(7R) | s s s s s s s d a | >90% | 31 |
| A.TL | s k k k k k k k d c | >90% | 25 |
| A.AL | k k k k k k k k d c | >90% | 21 |
| B10.D2 | d d d d d d d d d c | >90% | 33 |
| B10.A | k k k k k d d d d a | >90% | 30 |
| B10.AQR | q k k k k d d d d a | >90% | 37 |
| B10.A(4R) | b b b k k d d d d a | >90% | 39 |
| B10 | b b b b b b b b b b | >90% | 9 |
| D1.LP | b b b b b b b b b b c?/b? | >90% | 8 |
| B10.A(4R) | k k b b b b b b b b | >90% | 10 |
| B10.M | f f f f f f f f f f d | >90% | 0 |
| C58 | k k k k k k k k k a | >90% | 5 |
| B10.BR | k k k k k k k k k a | >90% | 2 |

T cells were prepared from spleen cells of strains shown using nylon fiber columns. Splenic non-T cells were prepared by pretreatment with anti-Thy-1 and C followed by isolation of the surviving cells on a Ficoll-Hypaque step gradient. The cytotoxic activity of MK-2.2 antibody was determined by preincubating T cells or non-T cells for 1 h at 0°C at 4 × 10⁶ cells/ml in neat culture supernatant taken from the hybridoma cells. The cells were washed several times and resuspended in 1:15 rabbit C. After 45 min at 37°C, viabilities were determined using trypan blue. The percent dead cells are shown after correction for cytotoxicity seen with C alone.

volume in 1:15 C (Microbiological Associates, Walkersville, Md.), and incubated at 37°C for 30 min. The cells were then centrifuged, washed once, and resuspended in culture medium.

Results

To test the idea that the activity of IL-2 as a B cell helper factor was dependent on its stimulation of the T cells contaminating most B cells prepared with conventional techniques, we set out to find a more effective means of T cell depletion. Our initial experiments (9) confirmed that IL-2 would stimulate an anti-SRBC response in spleen cells depleted of T cells by treatment with either monoclonal anti-Thy-1 or rabbit anti-T cell serum and C. Supplementing this treatment by injecting the spleen cell donors with ATS 2 d before killing and including α-MM in the B cell cultures dramatically reduced the stimulation seen with IL-2, indicating that the response depended on T cells contaminating the B cell preparation.

In more recent experiments, we found that supplementing the anti-Thy-1 and rabbit anti-T cell antibodies with the anti-Qa and Qa-like monoclonal antibodies B16-146, B16-165, and MK2.2 resulted in a treatment protocol that even more reliably resulted in a B cell preparation unable to respond to SRBC in the presence of IL-2 alone. Our rationale in using these additional antibodies was that because they were effective against a large proportion of Thy-1⁺ non-B cells, they might aide in the killing of both mature T cells that had low levels of Thy-1 and immature T cells (pre-T cells?) that might be stimulated to differentiate in culture to produce...
additional helper factors.

A comparison of the responses of T cell-depleted spleen cells prepared in this manner and spleen cells from nu/nu mice is shown in Table II. Both preparations failed to respond to SRBC without additional helper factors. Both responded well in the presence of normal Con A Sn. However, when using FS6-14.13 Con A Sn, only the nu/nu spleen cells responded. These results confirmed that IL-2 was a sufficient helper factor only in the presence of T cells and that conventional B cell sources such as nu/nu mice contained substantial T cell activity that could be revealed by IL-2.

The ability of IL-2 to enhance the activity of a small number of T cells was demonstrated even more dramatically in experiments in which various numbers of untreated spleen cells were added back to cultures of thoroughly T cell-depleted B cells in the presence of various helper factor preparations (Fig. 1). As before, these B cells cultured either with control medium containing Con A or with FS6-14.13 Con A Sn failed to respond to SRBC, although a response was obtained with normal Con A Sn. However, when as few as 1,000 normal spleen cells (<1%) were added to these cultures, a significant response was obtained with FS6-14.13 Con A Sn. Similar results were obtained using nylon fiber-purified splenic T cells (data not shown).

These results further demonstrated that for IL-2 to be a sufficient helper factor, cultures had to contain some T cell activity and point to the presence of additional helper factors in normal Con A Sn.

Both IL-2 and Another Factor(s) in Normal Con A Sn Are Required for B Cell Responses. Although our experiments established that IL-2 was not a sufficient helper factor for highly T cell-depleted B cells, they did not address the question of whether IL-2 was a necessary helper factor for B cells in the absence of T cells. The fact that normal Con A Sn stimulated responses even in thoroughly T cell-depleted B cells suggested that factors other than IL-2 might be the ultimate active principles and that all the effects of IL-2 might be mediated via T cells. If this were the case, we reasoned that the removal of IL-2 from normal Con A Sn should have no effect on its helper activity.

To test this prediction, we removed IL-2 from normal Con A Sn by incubation with IL-2-dependent T cells. We used Con A-activated normal spleen cells, T cells isolated from these Con A-activated spleen cells, and the IL-2-dependent cell line, HT-2, for this purpose. The results of these experiments are shown in Table III. In all

### Table II

**IL-2 Requires the Presence of T Cells to Be a Sufficient Helper Factor**

| B cell source | Helper factors | Total anti-SRBC PFC in 12 microcultures |
|---------------|----------------|----------------------------------------|
| nu/nu spleen cells | None | 0 |
| | FS6-14.13 Con A Sn | 629 |
| | Normal Con A Sn | 480 |
| Normal B6D2F1 spleen (ATS in vivo; anti-T cell cocktail + C in vitro) | None | 0 |
| | FS6-14.13 Con A Sn | 17 |
| | Normal Con A Sn | 625 |

Nu/nu spleen cells were cultured at 2.5 x 10^5 cells/microculture in medium containing nothing, 40% FS6-14.13 Con A Sn, or 30% normal Con A Sn. T cell-depleted B6D2F1 spleen cells were cultured at 1.5 x 10^5 cells/microculture in medium containing nothing, 40% FS614.13 Con A Sn, or 40% normal Con A Sn.
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![Graph showing the relationship between untreated spleen cells and total PFC in 12 microcultures.]

**Fig. 1.** A small number of T cells renders IL-2 a sufficient helper factor. T cell-depleted spleen cells were prepared from B6AF1 mice, as described in Materials and Methods. Untreated spleen cells from normal B6AF1 mice were also prepared. Various mixtures of these two cell preparations were cultured (keeping the total cell concentration at $1.3 \times 10^6$ cells/culture well) in medium containing 30% FS6-14.13 Con A Sn (●), 30% normal Con A Sn (▲), or 30% medium containing 4 µg/ml Con A (▲). All cultures received α-MM to a final concentration of 10 mg/ml and SRBC as antigen. Anti-SRBC PFC were assayed 4 d later. Results are reported as the total PFC in twelve culture wells vs. the fraction of untreated normal spleen cells in the culture. Similar results were obtained in two additional experiments.

cases, the IL-2-depleted Sn lost its ability to support the response of T cell-depleted B cells. In each case, FS6-14.13 Con A Sn, although unable to support a response by itself, restored the helper activity of the absorbed Sn.

To help establish that these absorptions resulted in specific absorption of IL-2 rather than in nonspecific destruction of various essential factors in the normal Con A Sn, we performed a number of control absorptions with various cell types other than IL-2-dependent T cells. These included normal spleen cells, LPS-activated B cell blasts, the T cell tumor line, BW5147, and a nonimmunoglobulin-secreting variant of

**Table III**

| Helper factor | Total anti-SRBC PFC in 12 microcultures |
|---------------|----------------------------------------|
|               | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 |
| None          | 0            | 0            | 0            | 0            |
| Normal Con A Sn* | 327          | 373          | 223          | 156          |
| FS6-14.13 Con A Sn‡ | 5            | 19           | 25           | 5            |
| IL-2-depleted normal Con A Sn*§ | 19           | 0            | 0            | 0            |
| FS6-14.13 Con A Sn‡ + IL-2-depleted normal Con A Sn* § | 143          | 439          | 309          | 217          |

* Normal Con A Sn and IL-2-depleted normal Con A Sn concentrations: experiment 1, 40%; experiment 2, 60%; experiment 3, 30%, experiment 4, 20%.
‡ FS6-14.13 Con A Sn concentrations: experiment 1, 40%; experiment 2, 20%; experiment 3, 40%; experiment 4, 40%.
§ IL-2 depletion of normal Con A Sn: experiment 1, absorption with Con A-activated spleen cells; experiment 2, absorption with Con A-activated splenic T cells; experiment 3, absorption with HT-2 T cells; experiment 4, preparation of 4-d normal Con A Sn. See Materials and Methods for details. Each IL-2-depleted Sn contained <10 U IL-2/ml.
MOPC 21. These absorbed Sn were compared to Sn absorbed with either Con A-activated spleen cells or HT-2 in their ability to support either HT-2 growth and viability or the anti-SRBC response of T cell-depleted spleen cells. The results are presented in Table IV. None of the control absorptions removed IL-2 from normal Con A Sn, as judged by the growth of HT-2. Similarly, these absorptions had no effect on the ability of the Sn to support an anti-SRBC PFC response. However, as before, absorption with either Con A-activated spleen cells or with HT-2 cells removed both the T cell growth activity of the Sn and its ability to support an antigen-SRBC PFC response. Also as before, the addition of FS6-14.13 Con A Sn to the IL-2-depleted Sn fully restored the anti-SRBC PFC response.

The results of these experiments indicated that at least two helper factors were required in the B cell response. Both were present in normal Con A Sn. One was specifically absorbed by IL-2-dependent T cells and the other was not. The absorbable factor could be supplied by FS6-14.13 Con A Sn but the nonabsorbable factor could not. These findings strongly implicated IL-2 as an essential component in FS6-14.13 Con A Sn, but because of the controversy over the possible effects of IL-2 on non-T cells, we performed an experiment to try to strengthen this conclusion.

We tested Con A Sn derived from a bank of independently derived T cell hybrids chosen to contain a wide range of IL-2 concentrations determined by the support of HT-2 growth and viability. Table V lists the hybrids and the levels of IL-2 present in their Con A Sn used in this experiments. They ranged from 1,800 U/ml in the Con A Sn of FS7-59 down to no detectable IL-2 in a number of Sn. These Sn were titered in cultures containing T cell-depleted B cells, SRBC, and excess IL-2-depleted normal Con A Sn, and the anti-SRBC PFC were assayed on day 4. The results are shown in Fig. 2. No response was obtained with IL-2-depleted normal Con A Sn. Restoration of the response was related to the level of IL-2. As described in the legend to Fig. 2, these data were used to estimate the units of activity of these Sn in the anti-SRBC PFC response. These estimates are listed in Table V. A comparison of U/ml in the HT-2 growth assay vs. the anti-SRBC PFC response shows a high degree of correlation (r = 0.96). From this data, one can calculate that under the conditions of these two

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**Table IV**

*Effect of Absorption of Normal Con A Sn with Various Cell Types*

| Absorbing cell* | Activity of normal Con A Sn |
|-----------------|---------------------------|
|                 | Units/ml IL-2 assayed on HT-2 | PFC/12 microcultures‡ |
|                 | Alone | Plus FS6-14.13 Con A Sn |
| None            | 320   | 137 | 163 |
| Normal spleen cells | 230   | 153 | 236 |
| LPS-activated B cells | 450   | 123 | 236 |
| Con A-activated spleen cells | <10   | <12 | 152 |
| HT-2            | <10   | <12 | 238 |
| MOPC-21         | 320   | 104 | 210 |
| BW5147          | 230   | 197 | 249 |

* See Materials and Methods for details.
‡ B cells from B6D2F1 mice. Normal Con A Sn, 30%. FS6-14.13 Con A Sn, 0.5% of a 50-fold concentrate. Response of B cells alone, <12 PFC. Response of B cells plus FS6-14.13 Con A Sn, 13 PFC.
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Table V
Correlation between the T Cell and B Cell Stimulator Activities of IL-2
Produced by Various T Cell Hybrids

| Hybrid  | Units of IL-2/ml in hybrid Con A Sn* | HT-2 growth assay† | B cell PFC assay§ |
|---------|-------------------------------------|--------------------|-------------------|
| FS7-59  | 1,800                               | 444                |                   |
| FS6-14.13 | 640                                   | 100                |                   |
| FS8-4   | 320                                  | 27                 |                   |
| FS8-14  | 80                                   | 18                 |                   |
| FS8-1   | 40                                   | <13                |                   |
| FS7-8   | <10                                  | <13                |                   |
| FS8-5   | <10                                  | <13                |                   |
| FS8-7   | <10                                  | <13                |                   |
| FS8-16  | <10                                  | <13                |                   |

* Hybrid cells incubated at 5 × 10⁵ to 10 × 10⁵ cells/ml for 24 h with 4 μg/ml Con A.
† As described in Materials and Methods.
§ Calculated from data in Fig. 3.

assays, 1 unit of IL-2 measured in the PFC response is equal to ~6 ± 3 units of IL-2 in the assay for T cell growth.

Taken together, these results make a very strong case for the identity between IL-2, defined as the lymphokine that supports the growth of continuous T cell lines, and

Fig. 2. Ability of Con A Sn of various T cell hybrids to restore IL-2-depleted normal Con A Sn. Microcultures were prepared containing 1.5 × 10⁸ T cell-depleted B6D2F₁ spleen cells, SRBC, α-MM, and 1 μl of 30 × concentrated IL-2-depleted 4-d normal Con A Sn. Cultures then received various amounts of Con A Sn from one of the following T cell hybrids: FS7-59 (●), FS6-14.13 (▲), FS8-4 (●), FS8-14 (□), or FS7-8, FS8-1, FS8-5, FS8-7, or FS8-16 (○). Cultures were assayed for anti-SRBC PFC on day 4. The results are shown as the total number of anti-SRBC PFC in 12 identical microcultures vs. the amount of T cell hybrid Con A Sn added per microculture. Also shown is the response of control cultures that received 40 μl of normal Con A Sn nor depleted of IL-2 (475 PFC/12 microcultures) and the estimated minimum detectable response (1 PFC/microculture or 12 PFC/12 microcultures). A unit of activity was defined as the amount of hybrid Con A Sn per microculture that stimulated 75 PFC/12 microcultures. (Halfway between 475 PFC and 12 PFC on the log₁₀ scale). Because 40 μl was the most Sn that could be tested, the preparations with <1U/75 μl or 13 U/ml fell below the limit of detection. The units of activity/ml estimated for each of the hybrid Con A Sn are listed in Table V.
the factor in FS6-14.13 Con A Sn that together with a second factor(s) in IL-2-depleted normal Con A Sn supports the anti-SRBC response of severely T cell-depleted B cells.

**Sephadex G-100 Chromatography of IL-2-depleted Normal Con A Sn.** To begin to identify the components in IL-2-depleted normal Con A Sn, which with IL-2 were required for the anti-SRBC B cell response, we fractionated the absorbed Sn on Sephadex G-100. Fractions were assayed for their ability to support the anti-SRBC response of T cell-depleted B cells in the presence or absence of added IL-2 Sn. The results are shown in Fig. 3a. None of the fractions of the absorbed normal Con A Sn were able to support a response. However, when tested in the presence of FS6-14.13 Con A Sn, which had little activity when tested alone, activity was revealed in the post bovine serum albumin fractions. The peak of this activity had an estimated ~40,000 mol wt.

In a reciprocal experiment (Fig. 3b), FS6-14.13 Con A Sn was fractionated on the same Sephadex G-100 column. The fractions were assayed both for their T cell-

![Figure 3](image-url)

**Fig. 3.** Sephadex G-100 filtration of IL-2-depleted normal Con A Sn and IL-2 Sn. A Sephadex G-100 column was prepared of approximate dimensions of 2.5 × 100 cm and equilibrated with 0.1 M ammonium bicarbonate. 7 ml of either normal Con A Sn that had been IL-2 depleted by incubation with Con A-activated spleen cells (panel a) or FS6-14.13 Con A Sn (panel b) were applied and eluted with 0.1 M ammonium bicarbonate. 19-ml fractions were taken, frozen, and lyopholized to remove water and ammonium bicarbonate. The residues were resuspended in 3 ml of medium. In panel a, each fraction was tested at 40% either alone (○) or with 40% FS6-14.13 Con A Sn (●) in the response of T cell-depleted B6AF1 spleen cells to SRBC. In panel b, each fraction was tested at 30% either alone (○) or with 30% IL-2-depleted normal Con A Sn (●) in the same response. In addition, these fractions were assayed for IL-2 activity using HT-2 cells, as described in Materials and Methods (hatched bars). The results are reported either as the total anti-SRBC PFC observed in 12 identical cultures or as units of IL-2 vs. the elution position. The molecular weight scale was determined with gamma globulin, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c. The void volume and total volume of the column are indicated.
stimulating activity and their ability to stimulate an anti-SRBC response from T cell-depleted B cells with or without added IL-2-depleted normal Con A Sn. None of the fractions alone supported a B cell response. However, addition of IL-2-depleted Con A Sn, which failed to support a response by itself, revealed a helper activity with a peak molecular weight of ~40,000. The IL-2 activity measured by the support of HT-2 growth emerged in these same fractions.

Discussion

We continued our efforts to define those helper factors in normal Con A Sn that were active in stimulating B cell responses to SRBC. We demonstrated that when using as a B cell source spleen cells which had been thoroughly depleted of T cells, at least two factors in normal Con A Sn were required for a response. The first factor was indistinguishable from IL-2. Its level in the Con A Sn of FS6-14.13 and a number of other T cell hybrids was directly correlated with IL-2 levels determined by support of T cell growth. The activity could be removed from normal Con A Sn by incubation with IL-2-dependent T cells but not with various other types of cells. The activity had an apparent 40,000 mol wt as determined by Sephadex G-100 chromatography. The second factor(s) was not produced by FS6-14.13 and was not absorbable with IL-2-dependent T cells but also had an apparent molecular weight of ~40,000, as determined by Sephadex G-100 chromatography.

These results indicate that IL-2 is capable of influencing B cell responses without an intervening T cell. This is a somewhat controversial point. Formerly called T cell-growth factor, IL-2 was originally defined by its powerful growth-stimulating activity for activated T cells (7, 8, 21). Its helper activity in B cell responses was attributed to its ability to expand the activity of residual T cells in the B cell preparations (7, 8). Indeed, in support of this view, several laboratories have shown (22, 23) that T cell lines could be established from nude mice using the combination of antigen or mitogen activation and IL-2. Our findings and a previous study (9) confirmed that IL-2 required only a very small T cell contamination of B cells to appear as a sufficient helper factor and that no response was obtained if care was taken to deplete T cells thoroughly. However, our results presented here indicate that IL-2 has a T cell-independent effect on the B cell response as well. These findings are in agreement with those of Parker (24), Howard et al. (25), and Swain et al. (26), who have demonstrated that IL-2 Sn from FS6-14.13 can influence B cell responses in the apparent absence of T cells. It could be argued that FS6-14.13 produces two similar factors: IL-2 that is T cell specific and a second factor capable of B cell stimulation. None of our experiments or those of others (24–26) formally rule out this possibility; however, the extremely strong correlation between the T cell- and B cell-stimulating activities in our experiments argues persuasively for a direct role of IL-2 in B cell responses without ruling out the existence of additional B cell-specific lymphokines.

It is not yet possible from our experiments to determine the role of IL-2 in the B cell response. It is tempting to suggest a role for IL-2 as a B cell growth factor. The experiments of Parker et al. (24) in fact suggest this role. Our finding that high concentrations of LPS-activated B cell blasts fail to absorb IL-2 might argue against this possibility, but alternatively it might be that the requirement for IL-2 is dependent on the means of B cell activation (i.e., antigen vs. LPS). We have not as yet been able to generate enough antigen-activated B cell to attempt an absorption. Alternatively,
it might be that IL-2 is not a growth factor for B cells but has some other effect on the B cell response. Bifunctional hormones and mediators are not unprecedented in immunological and other biological systems.

We should stress that in the experiments reported here, although care was taken to deplete spleen cells of T cells, no attempt was made to remove adherent accessory cells, such as macrophages. Macrophage-produced interleukin-1 (IL-1) has been reported as a B cell-helper factor and its synergy with IL-2 has been observed (27–29). The demonstration in our experiments that the non-T cell absorbable factor in normal Con A Sn had an apparent 40,000 mol wt would seem to exclude its being IL-1, although it seems likely that in the absence of T cells and macrophages, B cells may require IL-1 as well as IL-2 and this third factor for an optimal response. In fact, in preliminary experiments using IL-1 derived from the macrophage cell line P-388D1 (30, 31), we have observed that it does not substitute for the non-T cell absorbable, 40,000 mol wt, required factor and that both these factors and IL-2 are required by T cell and macrophage-depleted B cells for an optimal response to SRBC.

There are a number of reports of helper factors that might be identical to this non-IL-2 factor, which we have demonstrated in normal Con A Sn. Watson and his colleagues demonstrated a factor that copurified with IL-2, whose activity was manifested in B cell responses but not in T cell growth assays. This factor was partially resolved from IL-2 using isoelectric focusing (7). For a number of years Schimpl, Wecker, and their colleagues (2) have been studying the activity of a factor that they term T cell-replacing factor (TRF). Although the preparations in their early experiments and similar preparations studied by other investigators undoubtedly also contained IL-2 and perhaps IL-1, in their more recent work (32) they reported the separation of TRF from IL-2 on the basis of its physicochemical properties. Swain et al. (26) have recently demonstrated a marked synergy between FS6-14.13-produced IL-2 and an IL-2-deficient Sn obtained from a longterm alloreactive T cell line. As the purification of these various preparations improves and factors are exchanged between laboratories, it seems likely that some of these activities will be identical, and the naming of interleukin-3 will be justified.

As a final point, we would like to reemphasize that our results demonstrate that the determination of the mode of B cell stimulation by helper factors depends not only on adequate sources of uncontaminated factors but also to as great a degree on B cell preparations uncontaminated with other cell types.

Summary

A helper factor(s) distinct from interleukin 2 (IL-2) was shown to be present in the concanavalin A-stimulated supernatant of normal mouse spleen cells (normal Con A Sn). Spleen cells thoroughly depleted of T cells required both IL-2 and this factor to produce antibody-secreting cells in response to sheep erythrocytes, although in the presence of IL-2 and a few T cells the requirement for the factor was less apparent. The factor had an apparent ~40,000 mol wt. The factor was found in normal Con A Sn that had been depleted of IL-2 by absorption with IL-2-dependent T cells and was absent from Con A-stimulated supernatants of the IL-2-producing T cell hybridoma, FS6-14.13.

These results indicate that multiple helper factors control the B cell response to antigen and that IL-2, in addition to its T cell growth promoting activity, plays a
direct role in B cell responses.

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