CONCANAVALIN A-INDUCIBLE, INTERLEUKIN-2-PRODUCING T CELL HYBRIDOMA*

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Immunologists have found it difficult to reach a concensus on the mediators of the activity of helper T cells. Both antigen-specific and nonspecific factors have been reported (1-7), which we have suggested operate at different stages in the B cell response (8-10). Most preparations of nonspecific factors have been produced under conditions of polyclonal T cell stimulation, most often using the T cell mitogen concanavalin A (Con A).[1] The production of active supernates from normal T cells generally requires the presence of Ia^+ adherent cells (11), therefore, the resultant supernates contain a variety of T cell and non-T cell products, which potentially could both stimulate and suppress the B cell response. The picture has been further complicated by the recent findings of Watson et al. (12-14), who have demonstrated that a major component of nonspecific T cell supernates is T cell growth factor (TCGF) or interleukin-2 (IL2), whose activity in helper assays they suggest is caused by its ability to stimulate the activity of the small number of T cells present in B cell preparations, rather than caused by its direct effect on B cells.

As part of our effort to sort out the number and mechanism of action of helper T cell mediators we have been attempting to produce T cell hybridomas, which secrete one or at least only a few different mediators. We report here the successful isolation of a cloned stable T cell hybridoma, FS6-14.13, which when stimulated with Con A produces a factor indistinguishable from IL2. Moreover, other helper factors present in the Con A-stimulated supernate of normal spleen cells (normal Con A Sn) are missing in the hybridoma Con A Sn. This conclusion was reached in experiments demonstrating that unlike normal Con A Sn, the hybridoma Con A Sn was dependent on both T cells an adherent cells in order to be active in stimulating B cell responses to the antigen sheep red blood cells (SRBC).

Materials and Methods

Mice. $B6D2F_1$ mice were either purchased from The Jackson Laboratory, Bar Harbor, Maine, or bred in our own facilities.

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[1] *Abbreviations used in this paper:* A, aminopterin; ATS, rabbit anti-mouse thymocyte serum; BSA, bovine serum albumin; C, complement; Con A, concanavalin A; Con A Sn, supernate of cells incubated for 24 h with Con A; H, hypoxanthine; IL2, interleukin-2; α-MM, α-methyl mannoside; OVA, ovalbumin; PFC, plaque-forming cell; Sn, supernate; SRBC, sheep red blood cells; T, thymidine; TCGF, T cell growth factor.

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Antigens and Mitogens and Other Reagents. SRBC from a single animal (number 220) were purchased from the Colorado Serum Co., Denver, Colo. Ovalbumin (OVA) and α-methyl mannoside (α-MM) were obtained from Sigma Chemical Co., St. Louis, Mo. Con A was obtained from Miles-Yeda Ltd., Elkhart, Ind. Hypoxanthine (H), aminopterin (A), and thymidine (T) were also obtained from Sigma Chemical Co. They were used in culture at a final concentration of 1×10^{-4} M, 4×10^{-7} M, and 1.6×10^{-5} M, respectively. Polyethylene glycol 1540 was obtained from the J. T. Baker Chemical Co., Phillipsburg, N. J. It was used for cell fusion at a concentration of 50% w/w in serum-free medium.

Cell Culture. All cells were cultured by modifications (15, 16) of the methods of Mishell and Dutton (17), except where noted.

Cell Preparations. Treatment with anti-T cell serum and complement (C), nylon fiber column isolation of T cells, and adherent cell depletion using Sephadex G-10 were all performed as previously described (3, 16, 18).

Production of Supernates. Normal Con A Sn was prepared as previously described (3). Briefly, B6D2F$_1$ spleen cells were cultured at 10^7/ml overnight in medium containing a 4 μg Con A/ml. Hybridoma and BW5147 tumor cell Con A Sn was prepared by culturing the cells at 10^6/ml overnight in medium containing 4 μg Con A/ml. Cells were removed by centrifugation, and all supernates were stored at −20°C until ready for use.

Antisera. Rabbit anti-mouse thymocyte serum (ATS) was purchased from Microbiological Associates, Walkersville, Md. In vivo, 0.4 ml of a 1:10 dilution was injected intraperitoneally 2 d before sacrifice. For use with rabbit or guinea pig C as a specific anti-T cell reagent this antiserum was absorbed with the B cell tumor lines CH-1 or XS63 as previously described (18). CH-1 was kindly given to us by Dr. Noel Warner, Albuquerque, N. Mex. AKR anti-C3H (anti-Thy 1.2) was prepared as previously described (19). The batch used in these experiments had been well characterized. When used with selected rabbit C, it killed the appropriate proportions of spleen, lymph node, and thymus cells. It had also been demonstrated to deplete Con A responsiveness and helper function without affecting lipopolysaccharide responsiveness or B cell function.

Plaque-forming Cell Assays. B cells cultured in conventional 0.5-ml cultures were assayed for anti-SRBC plaque-forming cells (PFC) by the slide modification of the hemolytic plaque assay (17, 20). B cells cultured in 0.1-ml microcultures were assayed *in situ* for anti-SRBC PFC (15).

Results

Production and Characterization of FS6-14.13. Normal B6D2F$_1$ T cells were fused with azaguanine-resistant BW5147 AKR tumor cells (obtained from the Salk Cell Distribution Center, La Jolla, Calif.), essentially by the methods of Kontiainen et al. (21). Briefly, 10^7 T cells and 10^8 tumor cells were fused for 2 min in 1 ml of serum-free medium containing 50% w/w polyethylene glycol. The mixture was then diluted over a period of 10 min to a volume of 50 ml and then incubated for an additional 10 min at 37°C. The agglutinated cell suspension was centrifuged and the pellet dispersed in medium containing 5% fetal calf serum and 10% nutrient cocktail (complete medium) (17). The cells were distributed in 92 wells of 24-well Linbro 76-033-05 tissue culture plates. After 24 h all wells were supplemented with HAT. Culture medium was partially replaced several times a week with HAT containing fresh medium. After 2–3 wk stable hybrid growth was seen in 23 wells. These were subcultured into medium containing HT only for several passages and then finally into unsupplemented complete medium. At this point approximately 1.5×10^6 cells from each of these cultures (FS6-1 to -23) were incubated overnight in 1.5 ml of culture medium with 4 μg Con A/ml and the adherent portion of 2×10^5 peritoneal cells (macrophages). The supernates of these cultures were tested for helper factors. 50 μl of each supernate was added to a set of 12 microcultures containing 2×10^5 T cell-depleted B cells in 50 μl of culture medium containing SRBC as antigen. After 4 d microcultures were

assayed *in situ* for anti-SRBC PFC. Negative control supernates were Con A-supplemented medium and supernate from macrophage cultures stimulated with Con A. The positive control supernate (Sn) was normal Con A Sn. The results are shown in Fig. 1. Only one of the hybrid Sn had any activity (FS6-14). This line was cloned at limiting dilution. The resultant clones were tested for factor production as above, and all were positive. One clone (FS6-14.13) was selected for further study. FS6-14.13 was established as a T cell hybrid by the presence of the Thy 1.2 antigen on its surface (Fig. 2).

We established the conditions necessary for the production of an active Sn from the hybridoma. Although as a precaution we originally used macrophages in the production of Sn, we found that they were not required and omitted them in subsequent experiments. Fig. 3 shows the results of an experiment in which various Sn were tested for helper activity. Incubation of the hybridoma cells with Con A produced an active Sn. Sn from the hybridoma produced without Con A were without activity. Likewise, medium containing Con A, Sn from BW5147 stimulated with Con A, and Sn from unstimulated FS6-14.13 to which Con A was added after the removal of the hybridoma cells were all inactive.

There are several points concerning the response of FS6-14.13 to Con A worth noting. These are demonstrated by the data presented in Fig. 4. After the addition of Con A no helper activity was present in the Sn until at least 4 h. No further increase in activity was seen after about 24 h. In this respect the hybridoma behaved similarly to normal T cells. However, unlike the case with the majority of normal T cells, the growth of the hybridoma was severely inhibited by the presence of Con A. That this

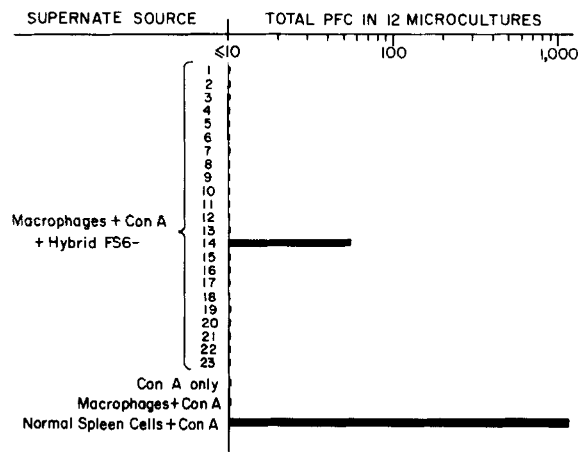

FIG. 1. Isolation of T cell hybridoma, FS6-14.13. Hybrids between B6D2F$_1$ T cells and BW5147 tumors cells were prepared as described in the text. Con A Sn were prepared with cells from the 23 culture wells that were positive for growth by culturing approximately 1.5×10^6 cells in 1.5 ml of culture medium containing 4 μg/ml Con A and the adherent portion of 2×10^6 peritoneal cells.

The Con A Sn were tested for helper activity as follows: for each supernate (Sn) a set of 12 microculture wells was prepared containing 50 μl complete medium, 50 μl Sn, 2×10^5 T cell-depleted spleen cells prepared from ATS-treated mice (17), and SRBC as antigen. After 4 d each microculture was assayed *in situ* for anti-SRBC PFC. The results are shown as the total PFC observed in a set of 12 identical wells. Negative control preparations were Con A-supplemented complete medium and a Sn from cultures containing Con A and macrophages only. The positive control Sn was normal Con A Sn. Only one of the hybrid Sn had any activity (from FS6-14). This line was cloned at limiting dilution and one of the clones (FS6-14.13) used in subsequent experiments.

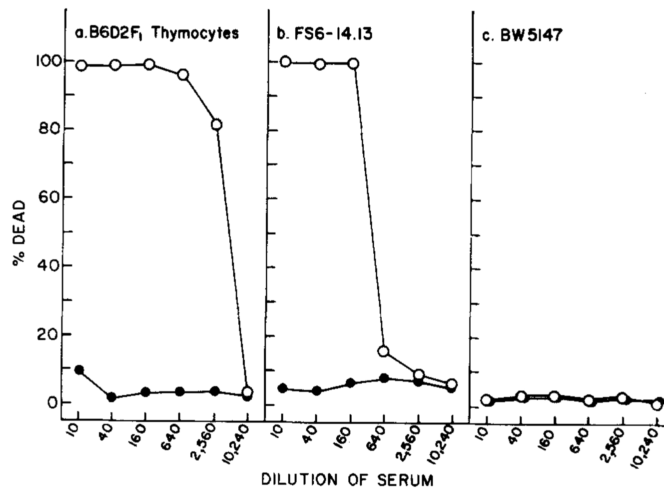

FIG. 2. Presence of Thy 1.2 on FS6-14.13. The cytotoxic titer of anti-Thy 1.2 (AKR anti-C3H) either before (○) or after (●) absorption with B6D2F1 thymocytes was determined on normal B6D2F1 thymocytes (a), FS6-14.13 (b), and BW5147 (c) as previously described (22), except that heat-inactivated fetal calf serum, rather than guinea pig serum was used as the dilutent for the rabbit C, which was used at a final concentration of 1:80. Results are shown as actual percent dead without correction for cell death with C alone.

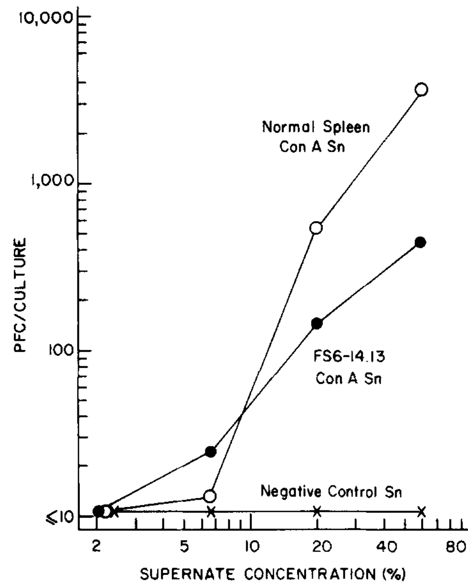

FIG. 3. Requirement for Con A stimulation in the production of helper factor by FS6-14.13. A Sn was prepared from FS6-14.13 cultured overnight at 10^6/ml in complete medium containing 4 μg Con A/ml (●). Other preparations (×) were Sn from BW5147 cultured with or without Con A, Sn from FS6-14.13 cultured without Con A, Sn from FS6-14.13 cultured without Con A and then supplemented with Con A after the removal of the cells, and complete medium supplemented with Con A. The positive control Sn (○) was normal Con A Sn. Various concentrations of each preparation were added to triplicate culture wells (Linbro 76-033-05) containing 2×10^6 T cell-depleted B6D2F1 spleen cells from ATS-treated mice and SRBC as antigen in a total volume of 0.5 ml. After 4 d identical culture wells were pooled and assayed for anti-SRBC PFC.

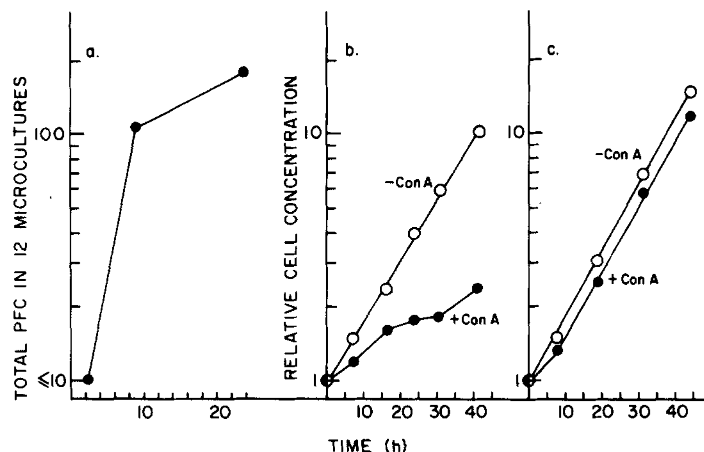

FIG. 4. Kinetics of factor production and effect of Con A on the growth of FS6-14.13. (a) Sn prepared from FS6-14.13 at various times after stimulation with Con A were tested at a concentration of 60% in microcultures as in Fig. 1. The results are shown as the total number of anti-SRBC PFC in 12 identical microculture wells vs. the time of the harvest of the Sn. In (b) FS6-14.13 cells were plated at 10^4/ml in complete medium with (●) or without (○) 4 μg Con A/ml. The cell concentration was determined at various times after the initiation of the culture. (c) represents similar growth curves for BW5147 cells.

property of the hybridoma was contributed by the normal parent was shown by the lack of effect of Con A on the growth of the parental tumor cell line BW5147.

Production of IL2 by FS6-14.13. Because the experiments of Watson et al. (12–14) had shown that a major helper factor in normal Con A SN was TCGF or IL2, we tested FS6-14.13 Con A SN for the presence of this activity assayed by its ability to maintain the proliferation of antigen-specific activated T cell blasts.

OVA-specific T cell blasts were produced as previously described (23). Briefly, OVA-primed lymph node T cells were cultured with OVA for 4 d. The T cell blasts were isolated and their growth continued for an additional 4 d in normal Con A Sn. Previous experiments had shown that at this point the blasts would continue their growth only in cultures containing either Con A Sn or OVA with irradiated splenic filler cells (23). Various preparations were tested for their ability to support the growth of these blasts. The results are shown in Fig. 5. Growth stopped abruptly in normal culture medium or in BW5147 Con A Sn. Vigorous growth was seen in cultures containing normal or FS6-14.13 Con A Sn. Other experiments have shown FS6-14.13 Con A Sn to be as effective as normal Con A Sn in supporting the growth of Con A-activated T cell blasts and two long-term helper T cell lines, HT-1 and HT-2 (kindly provided by Dr. James Watson, University of California, Irvine, Calif.) (data not shown). In addition, the presence of IL2 activity in FS6-14.13 Con A SN has been independently confirmed by Dr. Steven Gillis, Seattle, Wash. and Dr. Thomas Hünig, Massachusetts Institute of Technology, Cambridge, Mass. (S. Gillis and T. Hünig. Personal communications.).

Thus, it appeared that FS6-14.13 produced a IL2-like activity when stimulated with Con A. This conclusion was supported by the results of Sephadex G-200 chromatography. Samples of both normal and hybridoma Con A Sn were analyzed. The effluent fractions were assayed in both B cell and T cell stimulation assays. The

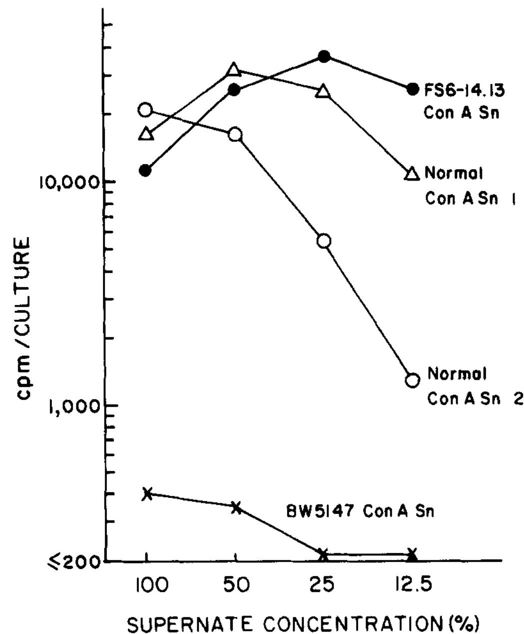

FIG. 5. Presence of IL2 activity in FS6-14.13 Con A Sn. OVA-specific T cell blasts were prepared as previously described (22). Microcultures were prepared containing various concentrations of either FS6-14.13 Con A Sn (●), BW5147 Con A Sn (X), or either of two batches of normal Con A Sn (O, Δ) (all supplemented with 20 mg/ml α-MM) and 10^4 OVA-specific T cell blasts. The cells were cultured for 4 d. During the last 24 h of culture, 1 μCi of [^{3}H]thymidine (sp act 6,700 μCi/μmol) was added to each culture. The incorporated ^{3}H was determined by liquid scintillation counting of the cells. Each point represents the average incorporated radioactivity in three replicate cultures. Similar results were obtained in numerous other experiments.

results are shown in Fig. 6. Although the total recovery of the T cell growth-stimulating activity was somewhat less than that of the B cell helper activity, for both Sn the activity in each assay emerged from the column shortly after albumin in the 30–40,000 mol wt range, consistent with results previously reported by this and other laboratories (3, 4, 12, 13, 24). There was a slight tendency with FS6-14.13 Con A Sn for the activity in the B cell assay to emerge before the activity in the T cell assay. Whether this difference represents heterogeneity in the factors in this Sn will have to be decided with more discriminating fractionation techniques.

Presence of Additional Helper Factors in Normal Con A Sn. The results presented in the previous section indicated that IL2 was a major component of FS6-14.13 Con A Sn. The question remained whether additional factors such as those previously reported to be present in normal Con A Sn were also present in the hybridoma preparations. The results of the experiments presented in Figs. 1, 2, 4, 5 already suggested a possible deficiency in the FS6-14.13 Con A Sn in that although this preparation was consistently stronger than normal Con A Sn in tests of T cell growth, it was weaker in assays for helper activity for B cells. We performed two additional experiments both of which indicated that IL2 was the only or at least the major helper factor in the hybridoma Sn, but that additional factors were present in normal Con A Sn.

In the first experiment we tested the prediction that the activity of IL2 in B cell responses was dependent on its stimulation of T cells contaminating the B cell

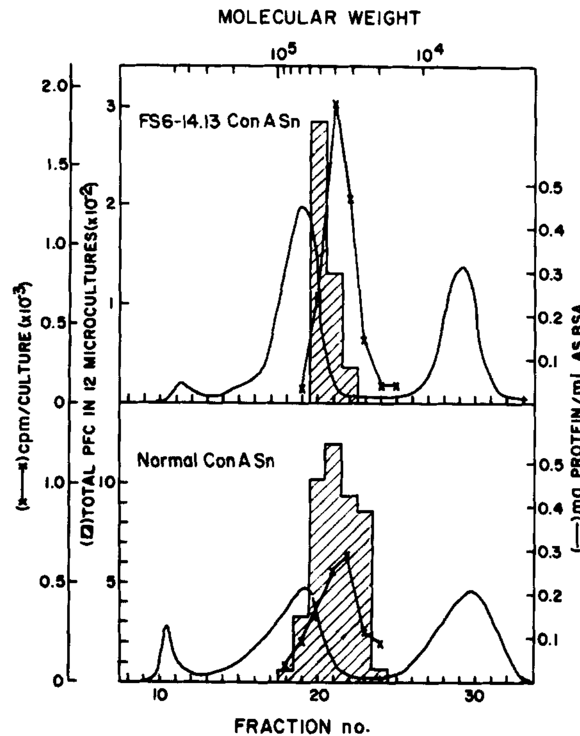

FIG. 6. Sephadex G-200 chromatography of FS6-14.13 and normal Con A Sn. A 100- × 2.5-cm Sephadex G-200 column was prepared and equilibrated with 0.1 M $(NH_4)HCO_3$. On sequential runs 7 ml of either FS6-14.13 or normal Con A Sn was applied to the column and eluted with 0.1 M $(NH_4)HCO_3$. 16-ml fractions were taken and thoroughly lyophilized removing both H_2O and $(NH_4)HCO_3$. The residues were resuspended in 4 ml of complete culture medium and tested for activity in both the response of T cell-depleted B cells to SRBC (▨) and the stimulation of growth in OVA-specific T cell blasts (X—X). In the anti-SRBC response the fractions were tested at 80% in microcultures prepared and assayed as in Fig. 1. The recovered activity from the column was approximately 75% for the FS6-14.13 Con A Sn and approximately 100% for the normal Con A Sn. In the test with OVA-specific T cell blasts, the fractions were supplemented with 20 mg/ml α-MM and tested at 50% as in Fig. 5. The recovered activity from the column was approximately 10–20% for the FS6-14.13 Con A Sn and approximately 30–50% for the normal Con A Sn. The protein concentrations of the eluates are shown (—) determined by absorbancy at approximately 280 nm with a bovine serum albumin (BSA) standard. The absorbancy in fractions 25–35 was caused primarily by phenol red and other low molecular weight compounds in the culture medium. The molecular weight scale was determined with the following markers: ferretin, IgG, BSA, chymotrypsinogen A, and cytochrome *c*.

preparation, but that normal Con A Sn contained additional factors that could stimulate the B cell response in the absence of T cells. A number of previous observations led to the design of this experiment.

We (18) and others (25), had shown that treatment of normal spleen cells with anti-T cell serum and C did not fully abolish helper T cell activity. Although cells prepared in this manner did not respond to SRBC, they contained enough T cells such that a good anti-SRBC response was obtained by the addition of Con A to the cultures. We demonstrated (18) that the bulk of these contaminating T cells could be removed by pretreatment of the spleen cell donor with ATS in vivo before the in vitro treatment with anti-T cell serum and C. Such preparations failed to respond to SRBC

even when Con A was added to the cultures. A further important observation was the finding that the activity of IL2 in stimulating T cells was dependent on their previous activation with either antigen or mitogen. IL2 was shown to be inactive on resting T cells (26).

Therefore, we designed an experiment in which normal and FS6-14.13 Con A Sn were compared in their helper activity under conditions where responsiveness to IL2, but not to factors directly reactive with B cells, should be selectively diminished. Namely, after reduction in the number of T cells by earlier treatment of the B cell donors with ATS, and/or after reduction in the activation of T cells by Con A present in the Sn (and consequently their susceptibility to IL2) by addition of α-MM to the culture.

As shown in Fig. 7, normal spleen cells treated with anti-T cell serum and C failed to respond to SRBC, but mounted a respectable response with the addition of mitogenic amounts of Con A (*a*). The efficacy of the methods to reduce T cell stimulation described above is demonstrated by the elimination of this ability of Con A to induce a response either by the pretreatment of the B cell donors with ATS (*b*) or by the addition of α-MM to the culture (*c*). Using cells treated with only anti-T cell serum plus C in vitro FS6-14.13 and normal Con A Sn produced equivalent responses. However, although either the addition of α-MM or pretreatment of the B cell donors with ATS dramatically reduced the response obtained in the presence of the hybridoma Sn, these procedures had a much less dramatic effect on the activity of the normal Con A Sn (*b* and *c*). Most importantly, the combination of ATS in vivo and α-MM in culture virtually eliminated the activity of the hybridoma Sn, whereas this combination had much less effect on the activity of normal Con A Sn (*d*).

We interpret these findings as an indication that the activity of FS6-14.13 Con Sn in B cell responses was dependent on the presence and activation of T cells, a property consistent with its containing IL2 as its primary, if not only, activity. Normal Con A Sn, on the other hand, clearly was sufficient for a response even under conditions of extremely low T cell activity, which suggests the presence of additional factors.

A second experiment supporting these conclusions stemmed from the observation by Hoffmann and Watson (27) that purified IL2 was ineffective in B cell responses in the absence of adherent cells. Because we had previously demonstrated that the activity of normal Con A Sn was not dependent on adherent cells (3), we compared the hybridoma and normal Sn for their activities in adherent cell depleted cultures. The results are shown in Fig. 8. Anti-T serum plus C-treated normal spleen cells responded equally well to both Sn as demonstrated above. Passage of the cells over Sepadex G-10 to remove adherent cells had no effect on the activity of the normal Con A Sn, as we previously reported. The procedure, however, totally eliminated the response obtained with the hybridoma Sn. This result was again consistent with IL2 being the predominant activity in the hybridoma Sn and again indicated the presence of additional activities in the normal Con A Sn.

Discussion

The cloned T cell hybridoma, FS6-14.13, clearly responded to Con A stimulation with the production of an activity thus far indistinguishable from TCGF or IL2. This factor could be assayed by its ability to stimulate the growth of activated T cells or to restore the response of partially T cell-depleted B cells to the antigen, SRBC.

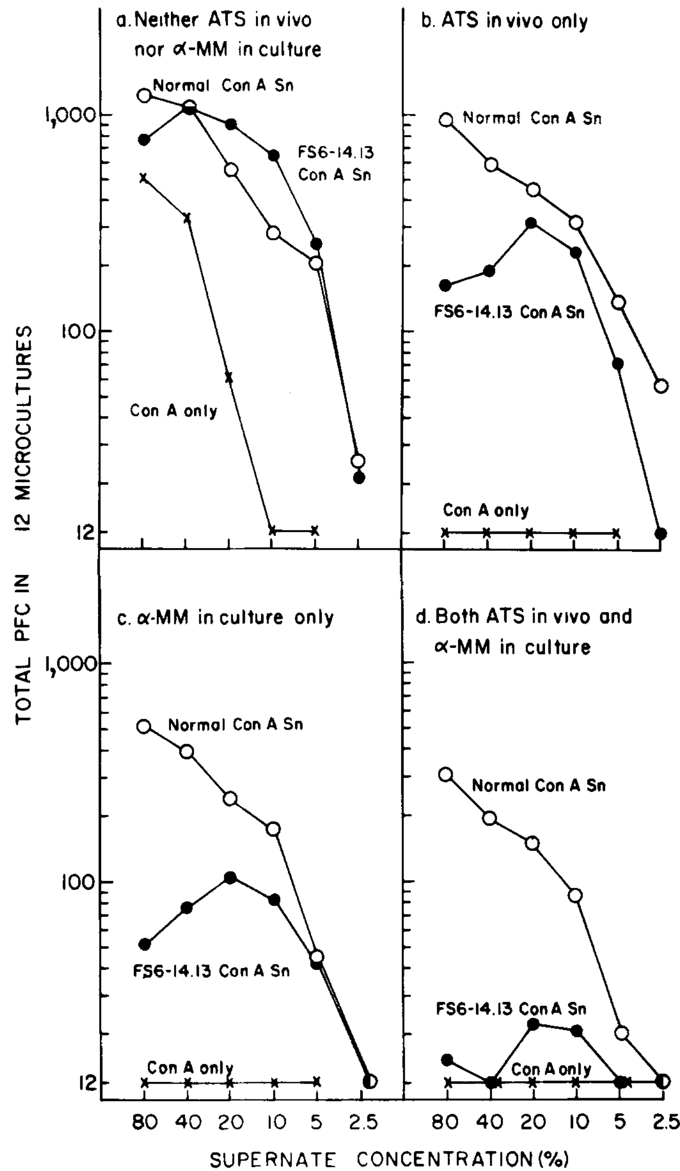

Fig. 7. Effect of thorough T cell depletion and Con A inhibition on the activity of FS6-14.13 and normal Con A Sn. T cell-depleted B cells were prepared by treatment of spleen cells from either normal (a and c) or ATS-pretreated (b and d) mice with anti-T cell serum and C. These cells were cultured at 1.5×10^5/microculture with SRBC as antigen. Cultures were supplemented with various concentrations of either medium containing 4 μg/ml Con A (X), FS6-14.13 Con A Sn (●), or normal Con A Sn (○). In addition, half the cultures (c and d) received 20 mg/ml α-MM. Results are shown as the total anti-SRBC PFC in a set of 12 identical microcultures on day 4. Similar results were obtained in two additional experiments.

The results reported here suggest that IL2 may be the only active principle present in the hybridoma Sn. Thorough T cell depletion of B cell preparations in combination with the prevention of T cell activation by Con A with α-MM eliminated the ability of the hybridoma to induce a B cell response. These procedures did not eliminate the

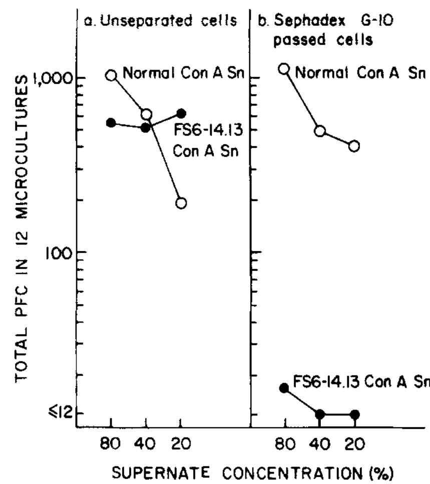

FIG. 8. Effect of adherent cell depletion on the activities of FS6-14.13 and normal Con A Sn. Spleen cells from normal mice were either left unseparated (a) or depleted of adherent cells by passage over a Sephadex G-10 column (b). Both preparations were treated with anti-T cell serum and C. These cells were cultured as in Fig. 7 with various concentrations of either FS6-14.13 (●) or normal (○) Con A Sn with SRBC as antigen. Results are presented as the total anti-SRBC PFC present in a set of 12 identical microcultures on day 4. Similar results were obtained in an additional experiment.

activity of normal Con A Sn, thus indicating the presence of activities in the normal Sn which were absent in the hybridoma Sn.

Depletion of adherent cells from the B cell preparation also eliminated the activity of the hybridoma Sn, without effecting the activity of normal Con A Sn. Whether this is an indication of essential helper factors produced by adherent cells as suggested by Hoffman and Watson (27) or whether this indicates the involvement of adherent cells in T cell activation is not distinguished by the results. However, this finding does support the IL2 nature of the active principle in the hybridoma Sn and offers further support for the conclusion that normal Con A Sn contains factors in addition to IL2.

Although our results indicate that IL2 is not sufficient for a B cell response in the absence of T cells, they do not show that IL2 is without effect on B cells. Results of experiments performed in collaboration with Dr. David Parker, Worchester, Mass. (28), indicated that even in the absence of T cells, the hybridoma Con A Sn dramatically stimulated the proliferation without antibody secretion of polyclonally activated B cells. Whether this represents the activity of IL2 or a second factor in the hybridoma Sn will have to be decided in future experiments.

The finding that normal Con A Sn contained helper activities in addition to IL2 supported the conclusions of a number of laboratories, who have reported multiple types of nonspecific helper factors (13, 24, 27). FS6-14.13 should prove useful as a source of one of these factors uncontaminated by the others. In addition this cell line represents a useful resource for the large scale production of IL2 for the continuous culture of T cell lines.

Summary

The fusion of an AKR T cell tumor line to normal $B6D2F_1$, T cells resulted in the production of a cloned T cell hybridoma (FS6-14.13) inducible with the mitogen

concanavalin A (Con A). The supernate from Con A-stimulated hybridoma cells was active both in the stimulation of an anti-sheep red blood cell response by partially T cell-depleted B cells and in the stimulation of the growth of antigen-specific T cell blasts. The active principle in both assays had a molecular weight of approximately 30-40,000. These results indicated the presence of interleukin 2 (IL2) in the hybridoma supernate.

The activity of the hybridoma supernate in B cell responses was dependent on the presence of adherent cells and a few contaminating T cells. On the other hand, Con A-stimulated supernates from normal spleen cells were active after either adherent cell removal or severe T cell depletion. These results suggested that IL2 was the only active helper factor in the hybridoma supernate, but that additional helper factors were present in supernates from Con A-stimulated normal spleen cells.

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