HELPER SIGNALS IN THE PLAQUE-FORMING CELL RESPONSE TO PROTEIN-BOUND HAPTENS*

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We have been interested in determining the mechanisms by which helper T cells collaborate with B cells in the generation of specific antibody responses. Based on a variety of experimental approaches we have proposed that the induction of B cell IgM plaque-forming cells (PFC)[1] responses to protein-bound haptens requires two types of helper T cell signals, the first both antigen specific and B cell Ia restricted and the second mediated by nonspecific factors, i.e. interleukins (1–6). Studies designed to test the synergistic effects of nonspecific factors and antigen-specific helper T cells have been complicated because the interleukins may have effects on T cell functions as well as, or instead of, the B cell response under study.

We previously established the ability of a series of T cell hybridomas to deliver antigen-specific, B cell Ia-restricted helper signals in the PFC response to soluble protein antigens (5). In addition to the obligate requirement for the antigen-specific helper T cell signal, the elicitation of optimal PFC responses was to varying degrees dependent on the addition of nonspecific factors provided by culture supernatants of concanavalin A-stimulated (Con A SN) spleen cells. This system offers an ideal opportunity to investigate the effects of nonspecific factors on B cell responses to soluble protein antigens under circumstances in which we are confident the effects of the factors are on the B cells themselves and not on the T cell hybridomas.

Our primary approach to studying the interleukins involved in the generation of specific B cell antibody responses has been the primary in vitro PFC response to sheep erythrocytes (SRBC). Unlike the antibody response to soluble proteins, spleen Con A SN are sufficient to drive the anti-SRBC PFC response of T cell-depleted splenic B cells (6). Using splenic B cells rigorously depleted of T cells and macrophages (MØ) we have been able to replace the activity of spleen Con

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[1] *Abbreviations used in this paper:* α-MM, alpha-methyl-D-mannoside; Con A, concanavalin A; Con A SN, culture supernatant of Con A stimulated cells; BCGF, B cell growth factor; FS6 SN, Con A SN of FS6-14.13; FS7 SN, Con A SN of FS7-20.6.18; HBSS, Hank's balanced salt solution; HGG, human gamma globulin; HRBC, horse erythrocytes; IaIf, Ia inducing factor; IFN_γ, interferon-γ; IL-1, interleukin 1; IL-2, interleukin 2; IL-X, interleukin X; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MØ, macrophage; MAF, MØ activating factor; PFC, plaque forming cell; P388 SN, culture supernatant of $P388D_1$; SRBC, sheep erythrocytes; TCGF, T cell growth factor; and TNP, trinitrophenol.

A SN with a combination of three nonspecific factor preparations (7–9). The first preparation is the culture SN of the MØ tumor cell line P388D_1 (P388 SN), the second the interleukin 2 (IL-2) containing Con A SN of the T cell hybridoma FS6-14.13 (FS6 SN) and the third, the interleukin X (IL-X) containing Con A SN of the T cell hybridoma FS7-20.6.18 (FS7 SN). These data suggested that at least three interleukins contribute to the PFC response to SRBC and that their effects can be mediated directly on the B cells themselves.

In this paper we demonstrate the synergy of these same three nonspecific factor preparations in the antigen-specific helper T cell-dependent PFC response to protein-bound haptens. These data confirm that antibody responses to SRBC and protein-bound haptens share similar nonspecific factor requirements, but that for responses to soluble protein antigens an antigen-specific H-2-restricted T cell activity is required as well.

Materials and Methods

Mice. B10.D2nSN, C57BL/6J, and C57BL/10SgSn mice were purchased from the Jackson Laboratory, Bar Harbor, ME. B6D2F_1 mice were bred in our facility from breeding stock obtained from the Jackson Laboratory.

Antigens and Other Reagents. Concanavalin A (Con A) was purchased from Miles Laboratories, Inc., Elkhart, IN; *Escherichia coli* lipopolysaccharide (LPS) from Difco Laboratories, Detroit, MI; 2-4-6-trinitrobenzenesulfonic acid (TNP) from Pierce Chemical Co., Rockford, IL; keyhole limpet hemocyanin (KLH) from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA α-methyl-D-mannoside (α-MM), and human gamma globulin (HGG) Cohn fraction II from Sigma Chemical Co., St. Louis, MO. Horse erythrocytes (HRBC) from a single animal were obtained from the Colorado Serum Co., Denver, CO. TNP-LPS was preparaed by the method of Rittenberg and Amkraut (10) as modified by Jacobs and Morrison (11). TNP-HGG and TNP-KLH were prepared as previously described (1). TNP-HRBC were prepared as described by Rittenberg and Pratt (12).

Immunizations. Splenic B cells were immunized for anti-TNP IgM responses by intraperitoneal injection of 10 μg TNP-LPS 7 d before use.

Cell Preparations. To prepare hapten-primed B cells depleted of both T cells and macrophages, TNP-LPS-primed mice were given an 0.4 ml injection of a 1:10 dilution of rabbit anti-mouse thymocyte serum (Microbiological Associates, Walkersville, MD) in Hank's balanced salt solution (HBSS), 3–4 d before use. Spleen cell suspensions from these mice were passed over Sephadex G10 columns (13), treated with a cocktail of anti-T cell antibody reagents for 30 min on ice, incubated with a 1:15 dilution of rabbit serum as the complement source (Grand Island Biological Co., Grand Island, NY) for 30 min at 37° C, washed, and then resuspended in medium. The antibody cocktail was similar to that previously described (14), containing rabbit anti-mouse thymocyte serum, T24/40.7 anti-Thy-1 monoclonal antibody (Dr. Ian Trowbridge, Salk Institute, La Jolla, CA), MK 2.2 anti-"Qa like" monoclonal antibody, B16/146 anti-Qat-4 ascitic fluid (Dr. Ulrich Hämmerling, Sloan-Kettering Memorial Cancer Institute, New York, NY), and ADH-15 anti-Lyt-2.2 ascites fluid (Dr. Paul Gottlieb, University of Texas, Austin, TX).

Cell Culture. 2×10^6 hapten-primed, T cell- and macrophage-depleted splenic B cells were cultured in 0.5 ml medium in Linbro 76-033-05 24-well culture plates (Linbro Chemical Co., Hamden, CT). Irradiated (1,300 rad) hybridoma T cells were added in the range of 0.5 to 2.0×10^5 per well. Culture conditions were modified from those of Mishell and Dutton (15, 16). Cultures contained 2.0 μg/ml TNP_7-HGG or 0.2 μg/ml TNP-KLH as antigen. To prevent effects due to residual Con A in nonspecific factor preparations, α-MM was added to all cultures at a final concentration of 10 mg/ml.

Assay for Plaque-forming Cells (PFC). On day four of culture, anti-TNP direct PFC were assayed by a slide modification of the Jerne hemolytic plaque assay using TNP-conjugated

HRBC (15).

T Cell Hybridomas. AODH7.1, AODK 10.4, and BDK 11.1 the antigen-specific, H-2-restricted T cell hybridomas used in these experiments were prepared as previously described (17, 18). AODH 7.1 is specific for the antigen HGG and I-E^d restricted; AODK 10.4 is specific for KLH and I-A^d restricted; and BDK 11.1 is specific for KLH and I-A^b restricted. The Con A-inducible T cell hybridomas FS6-14.13 and FS7-20.6.18 were prepared as previously described (8, 17).

Nonspecific Helper Factors. Culture supernatants of Con A-stimulated spleen cells (spleen Con A SN) were prepared as previously described (6). B6D2F_1 spleen cells at 10^7/ml in complete medium with 5% fetal calf serum, were cultured for 24 h with 4 μg/ml Con A. Cell-free SN were then harvested and stored at −20°C.

Macrophage-derived helper factor(s) were obtained as the constitutive culture SN of the MØ tumor cell line P388D_1 (P388 SN) (19). P388D_1 were cultured at 2×10^6/ml in complete medium containing 1% fetal calf serum. After 6 d of culture the cell free SN was harvested and stored at −20°C. This factor preparation has previously been shown to enhance the primary in vitro anti-SRBC PFC response of T cell- and MØ-depleted B cells (7). While we have not characterized the active component(s) of P388 SN in the anti-hapten PFC assay, the available evidence suggests that it is similar to the monokine interleukin 1 (20).

Interleukin 2 (IL-2) containing SN were prepared from the T cell hybridoma FS6-14.13 (FS6 SN) as previously described (14, 17). FS6-14.13 at 10^6/ml in complete medium containing 0.5% fetal calf serum were stimulated with 3 μg/ml Con A for 24 h. Based on the method of Watson et al. (21), the cell-free SN was made 0.1 M with α-MM and then $(NH_4)_2SO_4$ was added to 40% saturation. The resulting precipitate was removed and $(NH_4)_2SO_4$ added to the remaining SN to 80% saturation. The second precipitate was resuspended in 0.15 M NaCl and then extensively dialyzed against 0.15 M NaCl and then against HBSS. The overall concentration of the FS6 SN was ~50×.

The T cell growth-stimulating activity of FS6 SN was assayed using the IL-2-dependent T cell line HT-2, kindly provided by Dr. James Watson (Auckland, New Zealand; 14, 18, 22, 23). Cultures containing 100 μl twofold dilutions of SN were incubated for 24 h with 4,000 viable HT-2 cells. The first twofold dilution to yield <90% viable HT-2 cells was defined as containing 1 U IL-2. The concentrated FS6 SN used in these experiments contained 25,000–50,000 U IL-2/ml.

FS6 SN were depleted of IL-2 by incubation with HT-2 cells at 5×10^5/ml for 40 h at 37°C, at which time the SN contained <10 U/ml IL-2 (limit of detectability in HT-2 assay). The cell free SN was harvested and concentrated as described above.

Interleukin X (IL-X) containing factor preparations were Con A SN of the T cell hybridoma FS7-20.6.18 (FS7 SN). The Con A SN were prepared by stimulation of FS7-20.6.18 at 10^6 cells/ml with 4 μg/ml Con A in complete medium containing 10% fetal calf serum. After 24 h the cell free supernatants were harvested and stored at −70°C.

Con A stimulation of FS7-20.6.18 has been shown to lead to the production of interferon-γ (IFN_γ), MØ-activating factor (MAF), Ia-inducing factor (IaIF), and the B cell helper factor IL-X (8). The coordinate expression of these four lymphokine activities in subclones of FS7-20, their pH 2 sensitivities, and their frequent independent segregation from the production of IL-2 suggests either IFN_γ, MAF, IaIF, and IL-X are all activities mediated by a single factor or alternatively, two or more of these activities are mediated by different factors, the production of which is controlled by a common genetic mechanism (8). As a point of clarification, the functional activities of FS7-20.6.18 are not to be confused with FS7-6.18, an independent T cell hybridoma prepared in our laboratory, whose activities have been described by Isakson et al. (24).

Based on titration curves relating the virus agglutination titer to the log of the SN volume (25), FS7 SN contained 480 international units IFN/ml as compared with a National Institutes of Health (NIH) reference β-interferon (#G-002-904-511). FS7 SN contained <10 U/ml IL-2 as defined in the HT-2 assay (see above).

FS7 SN sensitivity to pH 2 neutralization utilized the method of Klein et al. (26). SN

were dialyzed for 24 h against 0.1 M glycine-HCl, 0.15 M NaCl, pH 2.0 buffer, followed by dialysis for 48 h against HBSS, pH 7.2 and then Mishell-Dutton media.

Results

Previous studies demonstrated the ability of the T cell hybridoma AODH 7.1 to deliver an HGG-specific, I-E^d-restricted helper signal in the anti-TNP PFC response of hapten-primed, T cell-depleted B10.D2 B cells and MØ-stimulated with TNP-HGG (5). In this system significant anti-TNP PFC responses were elicited in the absence of added nonspecific factors; however the addition of spleen Con A SN substantially increased these responses. We subsequently observed that the elicitation of anti-TNP PFC to TNP-HGG, driven by AODH 7.1, could be rendered completely dependent on the addition of the nonspecific factors in spleen Con A SN by using B cells depleted of MØ by G10 passage and rigorously depleted of T cells by treatment with anti-thymocyte serum in vivo and a cocktail of anti-T cell antibodies in vitro.

Synergy of Three Nonspecific Factor Preparations with the Antigen-specific Helper T Cell Hybridoma. Using this system we began analysis of the required nonspecific factors present in spleen Con A SN. Our approach was based on the previous demonstration that a combination of three nonspecific factor preparations could substitute for spleen Con A SN in the generation of a primary PFC response to SRBC (7–9). Fig. 1 demonstrates the ability of this combination of three nonspecific factor preparations; P388 SN, FS6 SN, and FS7 SN, to substitute for spleen Con A SN in the antigen-specific helper T cell-dependent PFC response to TNP-HGG. Three points are relevant to this data. First, no combination of factors substituted for the requirement for the antigen-specific helper signal provided by AODH 7.1. Second, in the presence of AODH 7.1, the generation of optimal anti-TNP PFC responses required the addition of all three nonspecific factor preparations, i.e. P388 SN + FS6 SN + FS7 SN and in combination, their activity was substantially greater than that of spleen Con A SN. Third, the anti-TNP PFC response elicited upon addition of all three factor preparations at optimal concentrations and times, was linearly related to the number of AODH 7.1 cells added.

Fig. 2 summarizes the available data regarding the relative contribution of each helper component. As mentioned above, the elicitation of PFC responses required the antigen-specific helper signal provided by the T cell hybridoma AODH 7.1 in conjunction with the nonspecific factors. In combination with AODH 7.1, each of the nonspecific factor preparations alone had a minimal effect on the generation of anti-TNP PFC responses to TNP-HGG. When the nonspecific factor preparations were used in paired combinations, significant PFC responses were observed that approached those elicited with spleen Con A SN. Comparison of the anti-TNP responses elicited with AODH 7.1 and paired combinations of the factor preparation suggests the relative stringency of the requirement for each preparation was P388 SN > FS6 SN > FS7 SN. When all three nonspecific factor preparations were used in combination, their activity was at least four times greater than that of spleen Con A SN or any of the paired combinations of factor preparations.

H-2 Restriction of Antigen-specific Help. In these studies we confirmed that

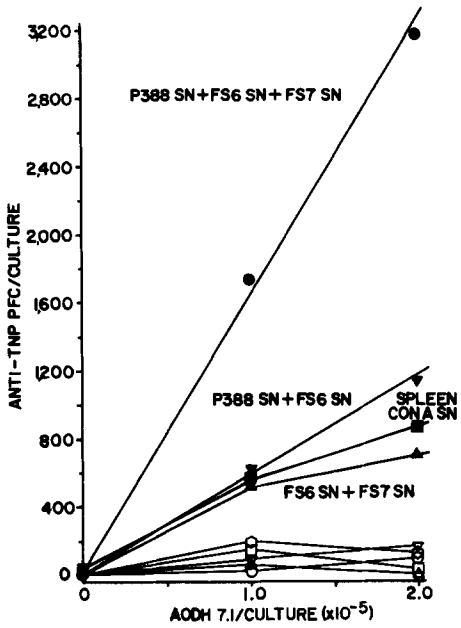

FIGURE 1. Titration of the HGG-specific, I-E^d-restricted helper T cell hybridoma AODH 7.1 in the presence of various combinations of the nonspecific factor preparations. Hapten-primed, T cell- and MØ-depleted B10.D2 B cells were stimulated with TNP-HGG in the presence of the indicated number of irradiated AODH 7.1 hybridoma cells and the indicated nonspecific factor preparations added at optimal times and concentrations. The anti-TNP PFC/culture were determined on day 4 of incubation. The combinations of factor preparations were: P388 SN + FS6 SN + FS7 SN (●), P388 SN + FS6 SN (▼), FS6 SN + FS7 SN (▲), P388 SN + FS7 SN (▽), P388 SN (□), FS6 SN (○), FS7 SN (○), spleen Con A SN (■), or nothing (△).

delivery of the antigen-specific helper signal by the T cell hybridomas, in the presence of the nonspecific factor preparations, was restricted by H-2 antigens expressed by the responding B cells. Two KLH specific hybridomas, AODK 10.4 which is I-A^d restricted and BDK 11.1 which is I-A^b restricted, were used as a reciprocal pair to test their requirements for H-2 identity with the hapten-specific B cells stimulated with TNP-KLH. As shown in Fig. 3, the I-A^d-restricted hybridoma AODK 10.4 stimulated the hapten-specific PFC responses by H-2^d B10.D2 B cells, but not H-2^b B10 B cells even in the presence of functional B10.D2 antigen-presenting cells. Conversely, the I-A^b-restricted hybridoma BDK 11.1 stimulated PFC responses by H-2^b B10 B cells, but not H-2^d B10.D2 B cells even in the presence of functional B10 antigen-presenting cells. In each case controls demonstrated that the presence of H-2 disparate B cell/MØ did not inhibit PFC responses generated by nonirradiated B cells and the appropriate T cell hybridoma. In addition, control experiments demonstrated the ability of the irradiated B cell/MØ to present KLH to the appropriate T cell hybridoma, although they were not able to develop into PFC. We concluded that in the presence of the three nonspecific factor preparations, delivery of the antigen-specific helper signal was restricted by H-2 antigens expressed on the hapten-specific B cells. Similar results have been obtained using AODH 7.1.

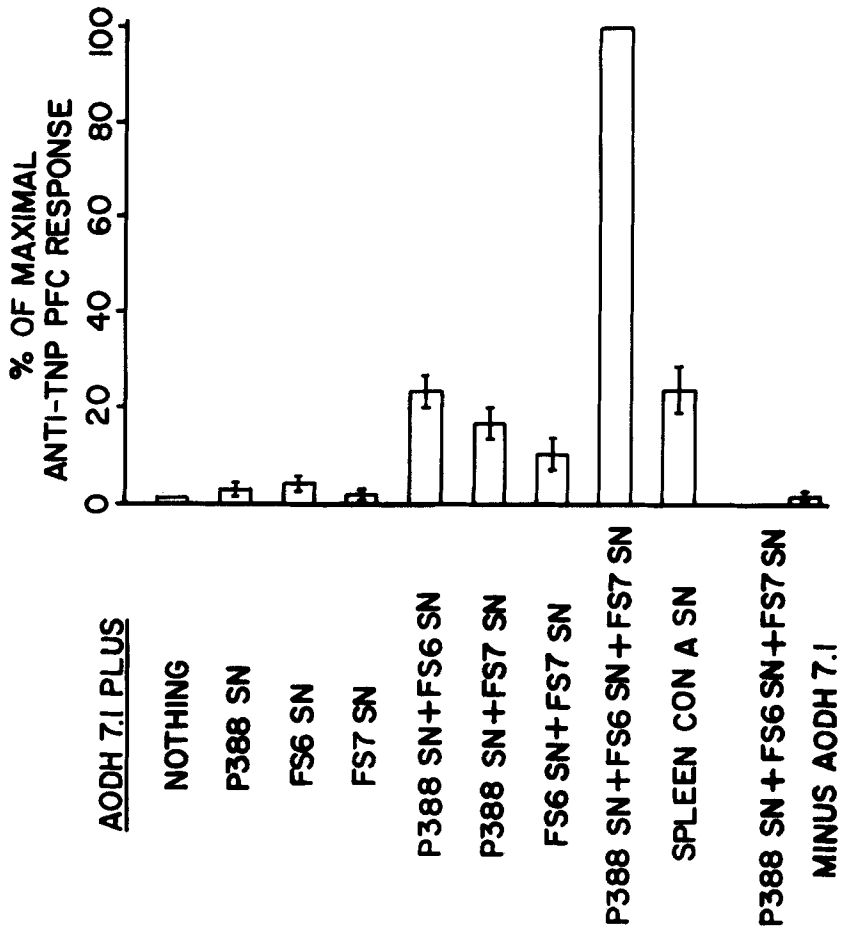

FIGURE 2. Ability of various combinations of nonspecific factor preparations to replace spleen Con A SN in the antigen-specific helper cell dependent PFC response to protein-bound hapten. Hapten-primed, T cell- and MØ-depleted B10.D2 B cells were stimulated with TNP-HGG in the presence of optimal concentrations of irradiated AODH 7.1 hybridoma cells (HGG, I-E^d). The indicated helper factor preparations were added at optimal times and concentrations. On day 4 of incubation the anti-TNP PFC/culture were determined and expressed as a percentage of the maximal response. Data summarize 12 experiments.

Requirement for Linked Recognition. In a representative experiment presented in Fig. 4 we demonstrate that in the presence of the three nonspecific factor preparations, delivery of the antigen-specific helper signal by AODH 7.1 required linked recognition of the hapten-carrier TNP-HGG. Stimulation with either free HGG or TNP conjugated to the irrelevant carrier KLH plus free HGG over the range of 2 to 1,000 μg/ml elicited marginal PFC responses as compared with TNP-HGG. The immunogenicity of the TNP-KLH in the PFC response of these B cells was demonstrated using the KLH specific hybridoma AODK 10.4. The HGG was also demonstrated to be immunogenic based on the IL-2 response of AODH 7.1 in the presence of irradiated B10.D2 spleen cells. We concluded that delivery of the HGG specific helper signal in the presence of the nonspecific

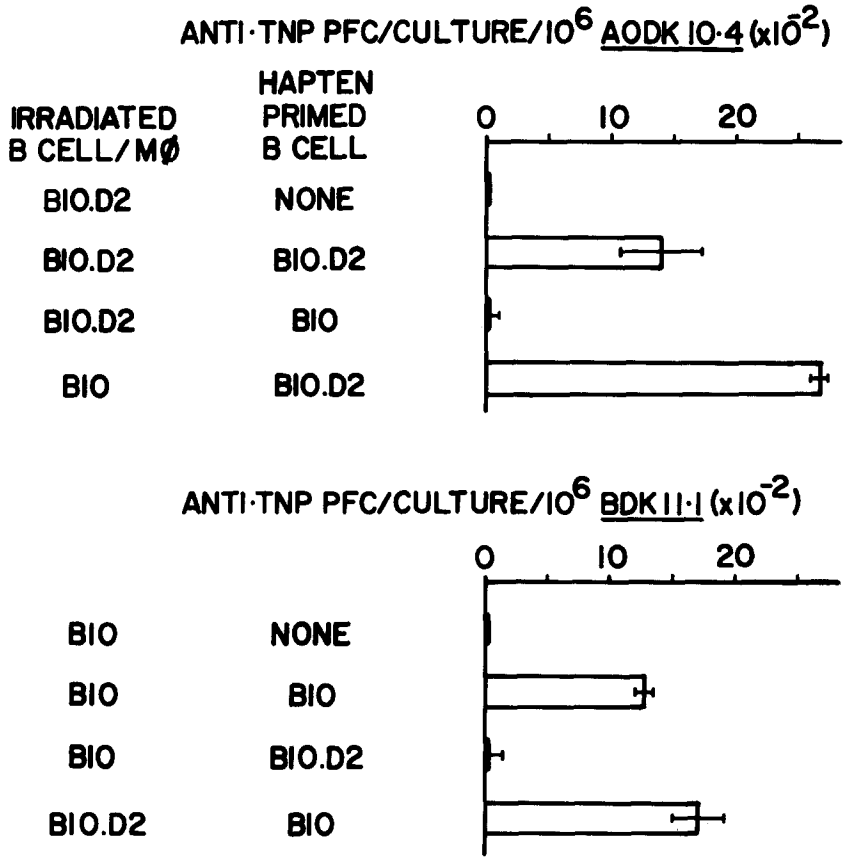

FIGURE 3. Delivery of the antigen-specific helper signal by T cell hybridomas in the presence of the nonspecific factor preparations was restricted by H-2 antigens on the responding B cells. Irradiated AODK 10.4 (KLH, I-A^d) or BDK 11.1 (KLH, I-A^b) were titrated into Mishell-Dutton cultures stimulated with TNP-KLH in the presence of optimal concentrations of P388 SN, FS6 SN, and FS7 SN nonspecific factor preparations. The responding hapten-primed, T cell- and MØ-depleted, B10 (H-2^b) or B10.D2 (H-2^d) splenic B cells were assayed for their anti-TNP PFC response in the presence of irradiated (4,000 rad) B10 or B10.D2 B cell/MØ as a source of antigen presenting cells. Control experiments demonstrated that the irradiated B cell/MØ were functional in the presentation of KLH to the appropriate T cell hybridoma. Data are expressed as the expected anti-TNP PFC per culture per 10^6 cells ± SE.

helper factors required linked recognition of the hapten-carrier conjugate.

Kinetic Analysis of Helper Component Addition. Kinetic analysis of the time of addition of each helper factor preparation in the primary anti-SRBC PFC response indicated that the interleukins present in P388 SN and FS6 SN act early in the response, while IL-X (provided by 4-d spleen Con A SN) was a "late" acting factor (7). We have reached similar conclusions with respect to the nonspecific factor requirements in the PFC response to protein-bound haptens. In the experiments summarized in Fig. 5, the time of addition of one helper component was varied and each of the remaining components were added at optimal times and concentrations. A 24-h or 48-h delay in the addition of P388 SN (panel *A*), FS6 SN (panel *B*) or the T cell hybridoma AODH 7.1 (panel *D*)

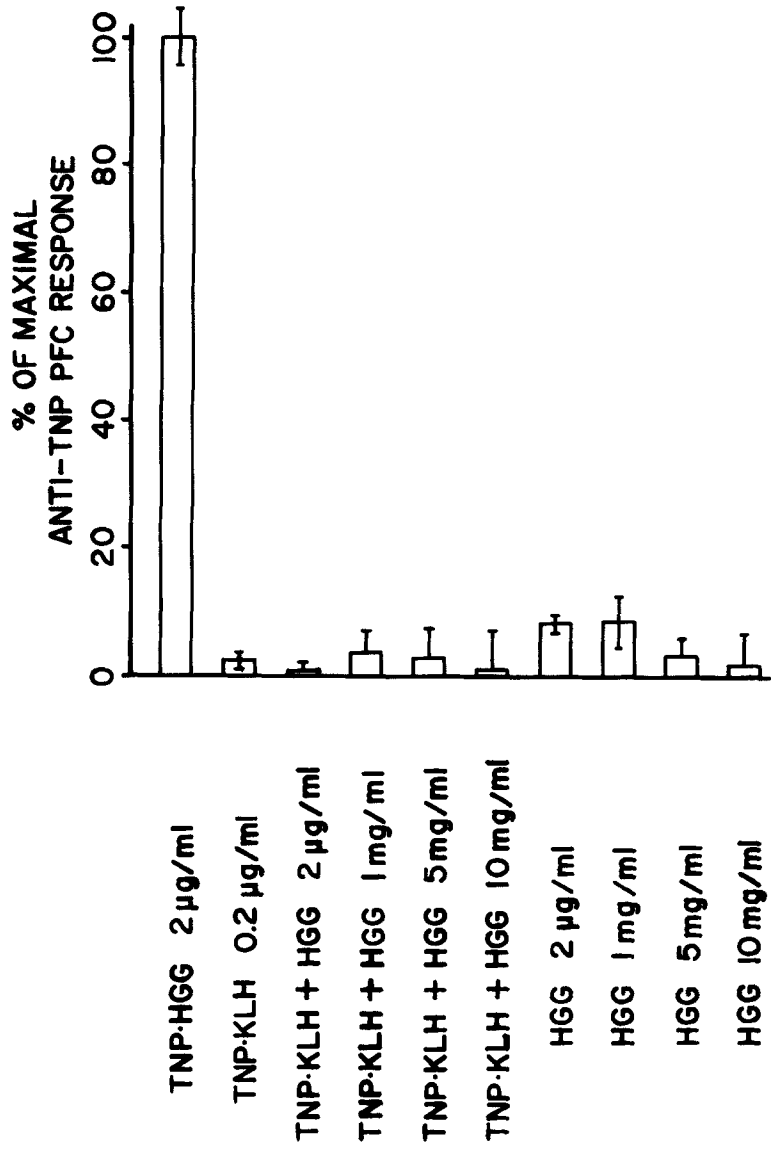

FIGURE 4. Requirement for linked recognition of hapten-carrier in the delivery of antigen-specific help by the HGG/I-E^d specific hybridoma AODH 7.1. Hapten-primed, T cell- and MØ-depleted B10.D2 B cells were stimulated with the indicated antigens in the presence of AODH 7.1 and optimal concentrations of the three nonspecific factor preparations: FS6 SN + FS7 SN + P388 SN. Data are expressed as a percentage of the optimal response elicited following titration of AODH 7.1 and calculation of the expected anti-TNP PFC/culture/10^6 hybridoma cells. Immunogenicity of TNP-KLH demonstrated using AODK 10.4 (KLH/I-A^d) and the same B cell pool (7,757 ± 1,299 anti-TNP PFC/culture/10^6 AODK 10.4 cells). Immunogenicity of HGG demonstrated using the IL-2 response of AODH 7.1 and irradiated B10.D2 spleen cells as antigen-presenting cells (1,280 U IL-2/ml at 1 mg/ml HGG).

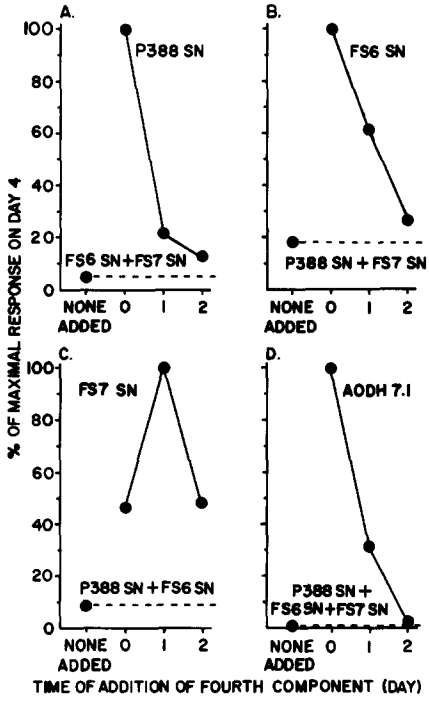

FIGURE 5. Kinetics of addition of helper component. In each panel a set of hapten-primed, T cell- and MØ-depleted B10.D2 B cells were stimulated with TNP-HGG in the presence of optimal concentrations of irradiated AODH 7.1 hybridoma cells (HGG, I-E^d). In panels *A*, *B*, and *C* each culture received two of the three helper preparations at optimal concentrations and times and the third factor preparation was either not added or added at optimal concentrations on day 0, 1, or 2. In panel *D*, each of the three factor preparations were added at optimal concentrations and times and the helper T cell hybridoma AODH 7.1 was either not added or added on day 0, 1, or 2. Anti-TNP PFC/culture were determined on day 4 of incubation. Following titration of AODH 7.1 the expected anti-TNP PFC/culture/10^6 hybridoma cells were calculated and the data expressed as a percentage of the optimal response. Data were pooled from two similar experiments.

significantly reduced the anti-TNP PFC response observed on day 4. In each case the longer the delay in addition of each of these helper components, the greater the reduction in the PFC response.

In contrast to the early requirement for the antigen specific helper signal provided by AODH 7.1 and the nonspecific helper factors in P388 SN and FS6 SN, the helper factor(s) provided by FS7 SN appeared to be "late" acting. Thus addition of FS7 SN at 24 h of culture resulted in significantly greater PFC responses than addition at the initiation of culture or at 48 h.

Titration of Nonspecific Factor Preparations. Further support for the conclusion that the independent effects of each nonspecific helper factor preparation were in fact due to qualitative rather than quantitative differences in the interleukins they contain is presented in Fig. 6. In each panel of Fig. 6 two of the three helper factor preparations were added at optimal concentrations (P388 SN 20%, FS6 SN 2%, and FS7 SN 20%) and times and the third factor preparation was titrated into the cultures at its optimal time of addition. Titration of each helper

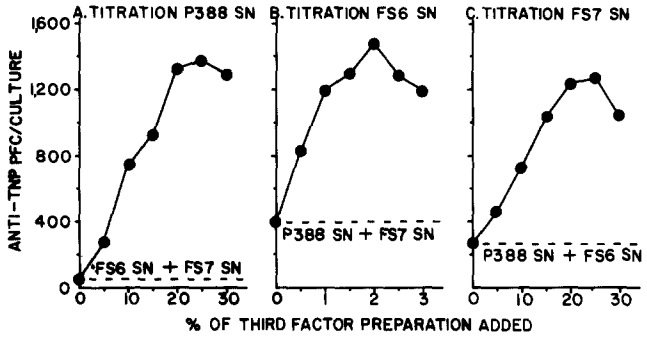

FIGURE 6. Titration of nonspecific factor preparations. In each panel a set of hapten-primed, T cell- and MØ-depleted B10.D2 B cells were stimulated with TNP-HGG in the presence of optimal numbers (1.5×10^5) of irradiated AODH 7.1 hybridoma cells (HGG, I-E^d). Each culture received two of the three nonspecific helper factor preparations at optimal concentrations (P388 SN 20%, FS6 SN 2%, and FS7 SN 20%) and the third preparation was titrated. Each factor preparation was added at optimal times. Cultures were assayed for anti-TNP PFC/culture on day 4 of incubation. Data were pooled from three similar experiments.

factor preparation confirmed that the concentrations used were in fact near saturation. These results substantiate the conclusion that even in the presence of optimal concentrations of two of the nonspecific factor preparations, the third factor preparation was required for a maximal response.

Characterization of Active Components in FS6 SN and FS7 SN. Independent studies have established that FS6 SN contained high concentrations of IL-2 as determined in a T cell growth factor (TCGF) assay and lack significant levels of IFN_γ as determined by the inhibition of virus replication, while conversely, FS7 SN contain high concentrations of IFN_γ and low levels of IL-2 (reference 8 and unpublished observations). In view of this information we attempted to establish further that the activity of FS6 SN and FS7 SN in the PFC response to protein-bound haptens were due to IL-2 and IFN_γ, respectively. These data are presented in Fig. 7.

Part of an aliquot of FS6-14.13 Con A SN was absorbed with the IL-2 dependent T cell line HT-2 and then concentrated by $(NH_4)_2SO_4$ precipitation in parallel with an unabsorbed control aliquot. The unabsorbed FS6 SN contained 25,600 U of IL-2 per ml and the HT-2 absorbed FS6 SN 400 U/ml. These two nonspecific factor preparations were titrated into cultures of hapten-primed B cells, AODH 7.1, P388 SN, and FS7 SN, all added at optimal concentrations and times. As shown in panel *A* of Fig. 7, IL-2 depletion of FS6 SN resulted in the loss of most but not all of its activity as compared to the unabsorbed FS6 SN control. The significant reduction in FS6 SN activity upon absorption with an IL-2-dependent T cell line suggests that IL-2 is one of the active components of this nonspecific factor preparation. The fact that the HT-2 absorbed FS6 SN had some residual activity has two possible explanations. First, AODH 7.1 is known to be capable of making IL-2 (18) although at this low antigen concentration, with this population of B cells as antigen-presenting cells and following irradiation, the ability of AODH 7.1 to make IL-2 is known to be substantially reduced if not abrogated. Second, FS6 SN may contain a second factor, which in addition to IL-2, is active in this assay system (see

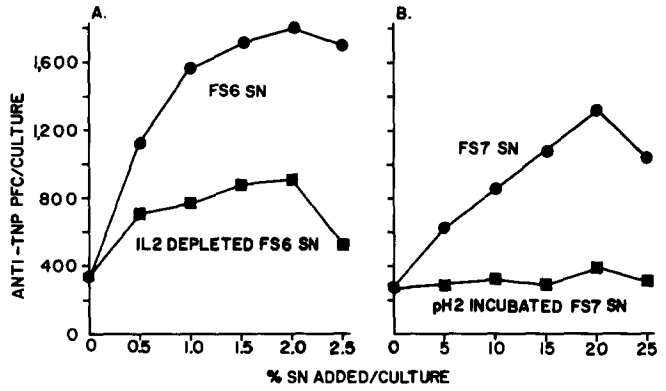

FIGURE 7. Characterization of active components in FS6 SN and FS7 SN. In panels *A* and *B*, hapten-primed, T cell- and MØ-depleted B10.D2 B cells were stimulated with TNP-HGG in the presence of optimal numbers of irradiated AODH 7.1 hybridoma cells (HGG, I-E^d). Panel *A*. Effect of IL-2 depletion of FS6 SN. Half of a pool of FS6-14.13 Con A SN was absorbed with the IL-2-dependent T cell line HT-2 as described in Materials and Methods. The IL-2-depleted SN was then concentrated in parallel with an unabsorbed control SN by $(NH_4)_2SO_4$ precipitation. The control and IL-2-depleted FS6 SN were then titrated into cultures containing P388 SN and FS7 SN added at optimal concentrations and times. Panel *B*. Sensitivity of FS7 SN to pH 2 incubation. Half of a pool of FS7-20.6.18 Con A SN was dialyzed against pH 2 glycine-HCl buffer and the other half dialyzed against pH 7.2 HBSS. Both of these SN were further dialyzed against HBSS and then culture medium. At 24 h of incubation these two preparations of FS7 SN were titrated into cultures containing P388 SN and FS6 SN added at optimal concentrations and times. On day 4 of incubation anti-TNP PFC/culture were determined.

discussion).

A characteristic feature of IFN_γ activity is its pH 2 sensitivity (26, 27). In an attempt to discern the pH 2 sensitivity of FS7 SN activity in the PFC response to protein-bound haptens, one half of an aliquot of FS7-20.6.18 Con A SN was dialyzed against pH 2 glycine-HCl buffer and the other half dialyzed against pH 7.2 HBSS. Each preparation was then further dialyzed against HBSS and then Mishell-Dutton media. The control and pH 2-incubated FS7 SN were titrated into cultures (at 24 h of incubation) of hapten-primed B cells, AODH 7.1, P388 SN, and FS6 SN, all added at optimal concentrations and times. As shown in panel *B* of Fig. 7, pH 2 incubation of FS7 SN abrogated its activity in the generation of anti-TNP PFC as compared to the dialyzed control FS7 SN. Mixing experiments (data not shown) established that the pH 2-incubated FS7 SN was not inhibitory in these cultures. These results support the conclusion that IFN_γ is an active component of FS7 SN in this system.

Discussion

On the basis of a variety of experimental approaches, we have proposed that antigen-primed T cells mediate two types of helper activities (1–6). One of these activities was first detected by the ability of antigen-primed T cells, upon specific restimulation, to nonspecifically drive the bystander B cell response to erythrocyte-bound antigens (1). This antigen nonspecific helper activity could be found in cell-free culture SN following specific challenge of antigen-primed T cells as well as following Con A stimulation of normal T cells (6). We have identified

three nonspecific factor preparations (FS6 SN, FS7 SN, and P388 SN) which in combination, will substitute for spleen Con A SN in the anti-SRBC PFC response of T cell- and MØ-depleted B cells (7–9). These results suggested that at least three interleukins act on B cells in the generation of PFC responses to SRBC.

In addition to nonspecific factors, a second type of activity, "antigen-specific help", was implicated in the PFC response to protein-bound haptens (1–6). While nonspecific factor preparations such as spleen Con A SN were sufficient to drive the anti-hapten PFC response to TNP-SRBC, they were insufficient in the response to TNP conjugated to proteins such as HGG or keyhole limpet hemocyanin. Based on a series of experiments utilizing limiting dilution, differences in the antigen dose response for priming and challenge, as well as differential fine specificity, we concluded that the two types of helper activity could be mediated by different T cells (1–3, 28). This conclusion received further support by the observation that anti-Ia sera raised against Con A blasts, eliminated a population of helper T cells whose function could be replaced by spleen Con A SN (4). Since spleen Con A SN was insufficient by itself, this again suggested that at least two types of helper function were involved at the effector phase in the PFC response to hapten-protein conjugates.

Subsequently we described the ability of a panel of T cell hybridomas to mediate antigen-specific help in the PFC response to protein-bound haptens (5). Delivery of the antigen-specific signal by the T cell hybridomas was carrier-specific and restricted by Ia antigens expressed on the hapten-specific B cells, suggesting that direct T cell/B cell contact was essential to deliver this helper signal. With a number of T cell hybridomas in the panel, the generation of anti-hapten PFC responses required the addition of nonspecific factors provided by spleen Con A SN. Since the hybridomas grow constitutively in the absence of exogenous growth factors, the requirement for nonspecific factors in addition to the carrier/I specific helper T cell hybridoma again supported the conclusion that two types of helper signals were required for this B cell response.

The present study attempted to determine the nature of the nonspecific factors required in the PFC response to protein-bound haptens. Our approach was based on the ability of three nonspecific factor preparations to fully replace the helper activity of spleen Con A SN in anti-SRBC PFC responses of T cell- and MØ-depleted B cells (7–9). The results presented demonstrate that the elicitation of hapten-specific PFC responses to TNP-protein conjugates by T cell- and MØ-depleted, hapten-primed B cells requires both an antigen-specific helper signal provided by a T cell hybridoma as well as nonspecific helper factors. In the absence of either of these helper activities, significant PFC responses were not observed. The elicitation of optimal anti-hapten responses required addition of all three nonspecific factor preparations: FS6 SN + FS7 SN + P388 SN.

In an attempt to confirm a qualitative requirement for each helper component in the PFC response to TNP-HGG, three of the components were added at optimal concentrations and times and the fourth was titrated into cultures at its optimal time of addition. The titration data (Figs. 1 and 4) show that each helper component was used near saturation and that in the presence of optimal concentrations of three of the helper components, the PFC response was directly related to the amount of the fourth component added. This result further substantiates

the conclusion that there was a qualitative requirement for at least three nonspecific factors in addition to an antigen-specific helper signal in the response to protein-bound haptens. Delivery of the antigen-specific helper signal in the presence of the three nonspecific factor preparations was shown to be both carrier specific and B cell I-region restricted.

The nonspecific factor requirements provided by the combinations of FS6 SN + FS7 SN + P388 DN, in the generation of optimal PFC responses to protein-bound haptens, directly parallel observations regarding the generation of anti-SRBC PFC responses. In addition kinetic experiments indicated that these three activities acted at similar times in both the anti-SRBC and the anti-TNP-protein responses. Thus the P388 SN had to be added very early to be effective. The FS6 SN was also required early, but the FS7 SN was optimal when added at day one of the 4-d response. The antigen-specific/I-region-restricted help was required from the initiation of the B cell response.

These experiments argue very strongly against the view that the helper T cell activity mediated via lymphokines and that mediated via direct antigen/I-region recognition by T cells on the B cell surface represent alternate helper pathways. Rather, they are consistent with our previously proposed model in which B cells may be activated either by efficient immunoglobulin cross-linking antigens such as SRBC or by T cell contact with antigen/I-region products on the B cell surface. B cells activated by either pathway become equally dependent on a set of lymphokines in order to complete their response to the point of antibody secretion.

The identity of the lymphokines in each of our factor preparations has not been established finally; however, we have performed some partial characterizations. It seems likely that the activity in P388 SN is IL-1 or IL-1-like molecules. We have not always found a direct correlation between the level of activity in these SN in our PFC responses *vs.* their activity in thymocyte proliferation assays, but G-75 chromatography of the SN has shown the active component in the SRBC response in fractions corresponding to 15,000 mol wt.

The Con A SN of the T cell hybridoma FS6-14.13 is known to have at least two lymphokine activities, T cell growth or IL-2 (17) and B cell growth factor (BCGF) (29, 30). It was previously inferred that IL-2 was an essential component of FS6 SN in the PFC response to SRBC on the basis of the ability of activated T cells or the IL-2-dependent T cell line HT-2, to deplete this B cell helper activity in parallel with TCGF activity (17). This conclusion was also supported by the coordinate expression of these activities both qualitatively and quantitatively in independent T cell hybridomas and their subclones. In addition, Sephadex chromatography of FS6 SN indicated that its IL-2 activity as measured by its ability to support the growth and viability of HT-2 cells, emerged at 30–40 kdaltons in the same column fractions as the B cell helper activity.

We have recently confirmed the presence of BCGF activity in FS6 SN, defining this activity similarly to Parker (29). As reported by Howard et al. (31) we have found the BCGF in FS6 SN to be ~15–20 kdaltons by Sephadex chromatography. The characterization of this activity in various PFC responses will be the subject of another report, but it is worth pointing out that partially purified BCGF is in our hands inactive in replacing IL-2 in anti-SRBC PFC responses,

nor have we yet found a synergy between IL-2 and BCGF. In the present study the preparation of concentrated, T cell-absorbed FS6 SN was virtually devoid of IL-2 but retained ~50% of its BCGF activity. Nevertheless it was unable at any concentration to replace the FS6 SN in the anti-TNP PFC response. This again indicated that IL-2 was one of the essential factors in the SN. It is worth noting, however, that after IL-2 removal this SN had more stimulatory residual activity than we would have predicted from our previous experience with anti-SRBC PFC responses. In fact this very batch of IL-2-deficient SN was without any residual activity in anti-SRBC PFC responses. It is possible that anti-TNP protein responses are more sensitive to BCGF than anti-SRBC responses, a possibility that can be tested after further purification of these two activities.

As has been the case for a number of years now, we are in a quandary to explain the role of IL-2 in B cell responses. There is no direct evidence that B cells can interact with IL-2 so that it has been widely suggested that IL-2 acts by revealing a hidden T cell activity in even the most scrupulously purified B cells. These and our previous studies would appear to eliminate BCGF and the factors in FS7 SN as the activities released by this contaminating T cell. Furthermore the current studies would seem to eliminate an antigen-specific/I-region-restricted B cell activating T cell as the target of the IL-2, since providing this cell in the form of an IL-2-independent T cell hybridoma did not alleviate the IL-2 requirement.

The available evidence suggests that the IL-X activity of FS7 SN in the anti-hapten PFC response is due to IFN_γ. In the present study this conclusion was supported by the pH sensitivity of its activity. This conclusion is further supported by extensive studies of the lymphokine activities associated with Con A SN of FS7-20.6.18 (8). The original hybridoma FS7-20 produced five lymphokine activities: IL-2, IFN_γ, MAF, IaIF, and the B cell helper activity IL-X. Analysis of a panel of clones and subclones of FS7-20 established that the ability to produce IL-2 frequently segregated independently from the other four activities. Surprisingly, the production of IFN_γ, MAF, IaIF, and IL-X in these subclones did not segregate independently and there was a high degree of correlation in the level of each lymphokine activity produced. This result suggested that either a single lymphokine mediated each of these functions or alternatively, two or more lymphokines were involved, with the genes coding for these lymphokines linked and coordinately expressed following Con A activation. Favoring the first interpretation was the observation that each of the lymphokine activities associated with FS7 SN was destroyed by pH 2 incubation. This conclusion has been further supported by independent studies demonstrating that preparations of IFN_γ potentiate in vitro and in vivo anti-SRBC PFC responses (32), and possess IaIF activity (33) and MAF activity (34). Conclusive identification of murine IFN_γ as a B cell helper factor in PFC responses to protein-bound haptens await its availability in pure form.

Summary

We have demonstrated the ability of a series of murine T cell hybridomas to deliver an antigen-specific, B cell I-region-restricted helper signal in the generation of specific PFC responses to protein-bound haptens. With some hybridomas

the elicitation of optimal PFC responses required the addition of nonspecific factors provided by culture supernatants of concanavalin A-stimulated (Con A SN) spleen cells. Using hapten-primed B cells depleted of both T cells and macrophages (MØ) we have now demonstrated a requirement for three nonspecific factor preparations to substitute for spleen Con A SN in the elicitation of optimal PFC responses. The first preparation was the interleukin 1 containing culture supernatant of the MØ tumor cell line P388D_1, the second the interleukin 2 (IL-2) and B cell growth factor containing Con A SN of the T cell hybridoma FS6-14.13, and the third, the gamma interferon containing Con A SN of the T cell hybridoma FS7-20.6.18. The P388D_1 and FS6-14.13 factor preparations were most effective when added at the initiation of culture, while the FS7-20.6.18 factor preparation was most effective when added at 24 h of culture. The activity of FS6-14.13 Con A SN was depleted by incubation with the IL-2-dependent T cell line HT-2. The activity of FS7-20.6.18 Con A SN was abrogated by incubation at pH 2. The results suggest that the generation of PFC responses to protein-bound haptens require at least three nonspecific factors in addition to an antigen/Ia specific helper signal.

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