ANTIGEN-SPECIFIC HELPER FACTOR IN MAN*

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Cooperation between thymus-derived (T)[1] lymphocytes and non-thymus-derived (B) lymphocytes has been shown to be necessary for the antibody response to protein antigens in experimental animals (1) and in man (2). Supernates of antigen-stimulated T cells can replace T cells for the in vitro antibody response by B cells (3). Two classes of T-cell helper factors have been demonstrated in experimental animals: nonspecific helper factors and antigen specific helper factors. Nonspecific T-cell helper factors (NSF) behave like polyclonal B-cell activators. They trigger autologous as well as allogeneic B cells to proliferate and secrete antibodies against a variety of antigens (4). On the other hand, antigen-specific helper factors (ASF) induce B cells to produce antibody that is directed exclusively against the antigen used to elicit the T-cell helper activity (5). In mice, ASF can be absorbed on immunosorbent columns containing the antigen used to raise the factor as well as on immunosorbent columns containing antisera to Ia determinants expressed on the cells that produced the factor (6). It has been thus suggested that ASF contains an antigen-binding site as well as determinants encoded for by the Ia region of the major histocompatibility complex.

Previous work in our laboratory has demonstrated that supernates of tetanus toxoid (TT)-stimulated human T cells contain a helper factor, termed lymphocyte mitogenic factor (LMF-TT) which induces proliferation, IgG synthesis, and specific anti-TT antibody synthesis in autologous as well as allogeneic B cells (2). Further work identified the presence of NSF in these supernates (7). It was shown that supernates depleted of TT antigen by passage over anti-TT immunosorbent columns were still capable of inducing proliferation and IgG synthesis in as much as 35–50% of B cells. Furthermore, NSF could induce specific antibody synthesis against TT as well as against an unrelated antigen such as diphtheria toxoid (DT) provided that second antigen was added to the T-cell cultures and provided the B-cell donor was immune to that antigen. The purpose of the present study was to identify ASF activity in the supernates of TT-stimulated human T lymphocytes.

Materials and Methods

Antigens, Antibodies and Enzymes. DT and TT were obtained from Massachusetts Biological Laboratories, Boston, Mass. They were purified by Sephadex G-200 chromatography (Phar-

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[1] *Abbreviations used in this paper*: ASF; antigen-specific helper factor; B lymphocyte, non-thymus-derived lymphocyte; DT, diphtheria toxoid; E, sheep erythrocytes; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; LMF, lymphocyte mitogenic factor; MI, mitogenic index; NSF, nonantigen-specific helper factor; PBS, phosphate-buffered saline; T lymphocyte, thymus-derived lymphocyte; TT, tetanus toxoid.

macia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). More than 90% of the material that eluted in the second protein peak was precipitable by specific rabbit antiserum. Iodination of TT and DT was carried with carrier-free iodine ^{125}I by the chloramine-T method (8).

Anti-TT and anti-DT antisera were raised in rabbits immunized with the antigen in complete Freund's adjuvant. Antibody titers to DT and TT were determined by hemagglutination of sheep erythrocytes coated with antigen using chromic chloride (9). They ranged from 1/8,000 to (and) 1/32,000. Rabbit anti-human IgG and anti-human IgM antisera specific to the heavy chain determinants were obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J.

Specific antisera for B-lymphocyte antigens (anti-DRW antisera) defined in the VIIth International Histocompatibility Workshop were used. Typing was performed using the standard methodology of that workshop (10).

Insolubilized ficin, amylase, and RNAase were purchased from Sigma Chemical Co., St. Louis, Mo. Insolubilized trypsin was obtained from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind. and insolubilized DNAase was obtained from Worthington Biochemical Corp., Freehold, N. J.

Anti-Idiotypic Antisera. Rabbit anti-idiotypic antiserum to anti-TT antibodies was raised in rabbits as described elsewhere.[2] Briefly, IgG from 1,000 ml of human plasma was purified by precipitation in 50% $(NH_4)_2SO_4$ followed by DEAE cellulose column chromatography. The purified IgG was digested with pepsin for 36 h at 37°C in a 0.1-M acetate buffer, pH 4.5. F $(ab')_2$ fragments were separated over a column of Sephadex G 150 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) then circulated over a TT-immunosorbent column. The material that bound to the column, F $(ab')_2$ anti-TT, was used to raise idiotypic antiserum in rabbits. The IgG fraction of the rabbit antiserum was rendered idiotype specific by extensive absorption against homologous TT nonreactive F $(ab')_2$. Anti-idiotypic antisera were shown to bind specifically to the immunizing F $(ab')_2$ anti-TT. High concentrations of antiserum bound >85% of ^{125}I-radiolabeled F $(ab')_2$ anti-TT but failed completely to bind to ^{125}I-radiolabeled TT nonreactive F $(ab')_2$. Studies with anti-idiotypic antisera have revealed minimal cross-reactivity, if any, between the idiotypic determinants of unrelated individuals because these antisera, even when used in high concentration, bound minimal amounts (<10%) of radiolabeled F $(ab')_2$ anti-TT derived from unrelated individuals. Furthermore, anti-idiotypic antisera were shown to inhibit the binding of ^{125}I-radiolabeled TT antigen to IgG derived from the donor of the immunizing F $(ab')_2$ anti-TT but inhibited poorly or not at all the binding of TT to IgG from unrelated donors. Anti-idiotypic antisera exhibited great specificity for the species of antibody molecules against which they were directed. Thus, anti-idiotypic antisera failed completely to inhibit the binding of ^{125}I-radiolabeled DT antigen to IgG derived from the donor of the immunizing F $(ab')_2$ anti-TT.

Immunosorbents. The IgG fraction was purified from serum by diethylaminoethylcellulose (DEAE 32, Whatman Chemicals, Maidstone, Kent, England) chromatography using a 0.01-M pH 6.8 phosphate buffer as eluting buffer. Proteins were dialyzed against 0.1 M $NaHCO_3$ and cross-linked to Sepharose 4B beads (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) activated by the cyanogen bromide method of Axen and Ernback (11). The efficiency of protein coupling to Sepharose beads exceeded 80% in all instances.

Preparation of Lymphocytes. Peripheral mononuclear cells were isolated from heparinized blood on Ficoll-Hypaque gradients (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) (12) or by means of a cell separator (Haemonetics, model 10, Haemonetics Corp., Natick, Mass.). 90% of the cells were mononuclear and viability exceeded 90% as determined by trypan blue exclusion. Cells were washed three times before use with Hank's balanced salt solution (Microbiological Associates, Walkersville, Md.) (HBSS).

Preparation of T-Cell-Rich Lymphocyte Suspensions. Lymphocyte populations rich in T cells were purified over nylon wool columns as described by Julius et al. (13). 10-ml plastic syringes were densely packed with 600 mg of extensively washed nylon wool (LP-1 Leukopak filter, Fenwal, Inc., Walter Kidde & Co. Inc., Ashland, Mass.) and sterilized by autoclaving. Before use, the

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[2] R. S. Geha, and R. P. Weinberg. 1978. Idiotypic antisera in man. Production and immunochemical characterization of idiotypic antisera to human anti-tetanus antibodies. *J. Immunol.* In press.

column was washed with HBSS containing 5% fetal calf serum (FCS). 50 million mononuclear cells in 2 ml of HBSS-FCS were applied to the column drop by drop. After a 45-min incubation period at 37°C, the nonadherent cells were washed out of the column with 50 ml of HBSS-FCS medium prewarmed to 37°C. 95–98% of the cells in the resulting suspension formed rosettes with sheep erythrocytes.

Preparation of Macrophage-Rich Populations. Mononuclear cells were suspended at a concentration of 1×10^7/ml in medium RPMI-1640 containing 10% FCS. 10 ml of cell suspension were incubated at 37°C for 1 h in a Falcon plastic tissue culture dish (BioQuest, BBL, & Falcon Products, Becton, Dickinson, & Co., Cockeysville, Md.). At the end of the incubation the nonadherent cells were sucked off and the cells adherent to the plates were washed three times with 10 ml of RPMI-1640 medium. The cells attached to the dish were then overlayered with calcium-free phosphate-buffered saline (PBS) and incubated for 30 min at 4°C. The bottom of the dish was then gently scraped with a sterile rubber policeman, the contents of the dish were spun at 200 *g*, and the cell button was resuspended in culture medium. The purity of the adherent cells as assessed by ingestion of 1.1 μm latex particles exceeded 93%.

Preparation of B-Cell-Rich Lymphocyte Suspensions. Lymphocyte suspensions rich in B cells were obtained by rosetting peripheral lymphocyte suspensions with sheep erythrocytes (E) pretreated with 50 U/ml neuraminidase (14) (Behring Diagnostics, American Hoechst Corp.) for 30 min at 37°C and sedimenting the lymphocyte erythrocyte mixture over a Ficoll-Hypaque gradient. The E non-rosette-forming cells which floated on top of the gradient were rerosetted and separated on another Ficoll-Hypaque gradient, and the final floating cells were used. They contained <2% E rosette-forming cells and 70–80% surface immunofluorescence positive cells when stained with a fluorescein conjugated polyvalent anti-human immunoglobulin antiserum.

Cell Cultures. Lymphocytes were cultured in medium 199 or RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% heat-inactivated human serum obtained from donors with type AB, Rh^+ erythrocytes, 50 U/ml penicillin G, and 50 μg/ml streptomycin, (complete medium). They were then placed in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air.

Production of T-Cell Supernates. Peripheral blood lymphocytes or alternatively T-cell-rich lymphocytes containing 5% macrophages were cultured at a concentration of 1×10^7 cells/ml in complete medium 199 for 48 h with antigen (TT 2–10 μg/ml). A duplicate set of cultures received an equivalent amount of antigen at the end of the incubation period and served as control. The incubation was terminated by pelleting the cells at 200 *g* for 10 min. Supernates were collected, filtered through a 0.45 μm filter (Millipore Corp., Bedford, Mass.), and stored at −20°C until tested.

Fractionation of T-Cell Supernates on TT-Immunosorbent Columns. 20 ml of T-cell supernates were circulated over immunosorbent columns of Sepharose 4B cross-linked to TT at a rate of 2 ml/h for 48 h at 4°C. The material that eluted spontaneously from the column was collected and designated NSF. This material was previously shown to induce proliferation, IgG synthesis, and anti-TT-antibody synthesis in both allogeneic and autologous B cells (7). The immunosorbent column was then washed extensively with PBS, pH 7.2 for 24 h. The material bound to the column was then eluted with 3 M NaCNS in a volume equivalent to the original volume of supernates applied to the column, was dialyzed extensively and successively against 0.15 M NaCl and medium RPMI-1640, filtered through a 0.45-μm filter, and stored in 1% human serum albumin at −20°C until tested. This material was designated ASF. Experiments made with the addition of ^{125}I-TT to LMF-TT before passage over the column demonstrated that <5% of the radioactivity was retained on the column. Control material was generated by fractionating the T-cell supernates over an immunosorbent column consisting of Sepharose 4B cross-linked to DT.

Fractionation of T-Cell Supernates on Anti-TT-Immunosorbent Columns. T-cell supernates were passed over an anti-TT immunosorbent column as described above. The material that eluted spontaneously from the column was depleted of antigen and was shown previously to induce proliferation and IgG synthesis in autologous, as well as allogeneic, T cells (7). The material that bound to the column, which included the TT antigen present in the T-cell supernates, was eluted with 3 M NaCNS and processed as described above. Experiments made with the addition of ^{125}I-TT to T-cell supernates before their passage over the column demonstrated that >85% of the radioactivity was retained on the column. Furthermore, analysis of 3 M

NaCNS washings from the column revealed no detectable anti-TT activity as determined by a radioimmunoassay utilizing ^{125}I-TT, with normal rabbit serum and goat anti-rabbit IgG at equivalence. Control material was generated by passing T-cell supernates over an anti-DT immunosorbent column.

B-Cell Proliferation. B lymphocytes were cultured in 0.2 ml cultures in the wells of microtiter plates (BioQuest, BBL, & Falcon Products, Becton, Dickinson, & Co.) at a concentration of 1 × 10^6 cells/ml and in the presence of 50% unfractionated or fractionated T-cell supernates. After a 6-day incubation period, the cultures were pulsed with [^{3}H]thymidine (New England Nuclear, Boston, Mass., sp act 2 Ci/mol) (0.5 μCi/culture) and harvested 16 h later with an automatic tissue culture harvester (Skatron, Flow Laboratories, Inc., Rockville, Md.). Results were expressed as counts per minute of [^{3}H]thymidine incorporated into DNA per culture and as mitotic index (MI) where:

$$\text{MI} = \frac{\text{cpm of } (^3\text{H})\text{thymidine incorporated in stimulated culture}}{\text{cpm of } (^3\text{H})\text{thymidine incorporated in nonstimulated culture}}.$$

Immunoglobulin Synthesis. B lymphocytes were cultured at 2 × 10^6 cells/ml in sterile culture tubes (BioQuest, BBL, & Falcon Products, Becton, Dickinson, & Co.) in the presence of 50% T-cell supernates. Every 36 h, one-half of the culture medium was removed and replaced with an equivalent amount of fresh culture medium. After 5 days of incubation, the cells were washed three times with HBSS and then resuspended at 4 × 10^6 cells/ml in fresh RPMI-1640 medium deficient in L-valine, L-leucine, and L-isoleucine, and supplemented with 2 μCi/ml ^{14}C-radioactive amino acids and with dialyzed fetal calf serum (Microbiological Associates, Walkersville, Md.). 48 h later, the B-cell culture fluid supernates were harvested, filtered through a 0.45-μm filter, and frozen at −20°C until tested. Newly made IgG, present in supernates of B-cell cultures, was determined by a sandwich radioimmunoassay as described previously (7). Briefly, 0.1 ml of supernates was incubated with 20 μg of human IgG and rabbit human IgG at equivalence at 37°C for 1 h and at 4°C overnight. The precipitates were washed three times with ice-cold PBS containing 1% human serum albumin and 0.5% Tween 20 (Atlas Chemical Industries, Inc., Wilmington, Del.). The final precipitates were dissolved in 0.5 ml of 0.5% acetic acid, transferred to scintillation vials containing 10 ml of Instagel counting solution (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in a Packard Tri Carb liquid scintillation counter (Packard Instrument Co., Inc.). IgG with anti-TT antibody activity was determined as follows. To 0.1 ml of B-cell supernates, 30,000 cpm (~10 ng) of ^{125}I-TT was added. After an initial 1-h incubation at 37°C, equivalent amounts of human IgG myeloma (devoid of anti-TT activity) and of rabbit anti-human IgG (preabsorbed with TT cross-linked to Sepharose 4B) were added. The precipitates were washed as described above and counted in a Packard gamma scintillation counter (Packard Instrument Co., Inc.). The amount of radioactivity precipitated was compared to the radioactivity precipitated with different dilutions of a standard IgG preparation of known anti-TT antibody content. ^{125}I-DT and rabbit anti-human IgG preabsorbed with DT cross-linked to Sepharose 4B were used in control experiments. Specificity of these radioimmunoassays was established by demonstrating that cold unlabeled antigen completely inhibited the precipitation of radiolabeled antigen.

Absorption of T-Cell Supernates with Antisera Cross-Linked to Sepharose 4B. 2-ml aliquots of unfractionated LMF-TT, NSF, or ASF were incubated with 0.5 ml of Sepharose 4B beads cross-lined, respectively, to the IgG fraction of antisera directed against human IgG, IgM, and B-cell alloantigens (Ia analogues). The incubation was carried in 12 × 75 mm Falcon plastic tubes for 6 h at 4°C with continuous rotation. The beads were then sedimented by centrifugation (5 min, 200 *g*), and the supernatant material was collected, millipore-filtered, and assayed on B cells.

Effect of Anti-DRW Antisera on B-Cell Cultures. Anti-DRW antisera were heat inactivated at 56°C for 45 min and added at a final dilution of 1:10 to B-cell cultures.

Treatment with Enzymes. 2-ml aliquots of ASF were incubated with the following insolubilized enzymes: 10 U trypsin, 5 U ficin, 20 mg amylase, 1,000 U DNAase, and 5 U RNAase. The incubation was carried at room temperature for 6 h, at the end of which the cross-linked enzymes were pelleted by centrifugation (5 min, 200 *g*) and the supernatant material was collected, millipore-filtered, and assayed on B cells.

TABLE I

Effect of LMF-TT, NSF, and ASF on Autologous B Cell Cultures

Helper factors	MI total IgG synthesis Δ(P-R) cpm ^{14}C-IgG*		IgG anti-TT synthesis Δ(P-R) ^{125}I cpm‡
LMF-TT	10.5	+9,120	+3,250
NSF + TT	8.2	+8,830	+2,764
ASF	1.1	−92	+21
ASF + TT	1.0	+130	+2,843

* cpm of ^{14}C-IgG secreted by 1×10^6 B cells.

‡ cpm of ^{125}I-TT precipitated by the supernates of 1×10^6 B cells.

Sephadex G-100 Gel Chromatography. A 1.5 × 100-cm Sephadex G-100 column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) maintained at 4°C was calibrated with different protein markers obtained from Schwarz/Mann Div. Becton, Dickinson, & Co., Orangeburg, N. Y.: Rabbit IgG (mol wt 150,000), bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 43,000), chymotrypsinogen A (mol wt 25,000), myoglobin (mol wt 17,800) and cytochrome *c* (mol wt 12,500). 10 ml of material of freshly isolated ASF were loaded into the column. The column was washed using PBS at a flow rate of 18 ml/min and 10-ml fractions were collected. Fractions were pooled according to desired molecular weight ranges determined by previous calibration then, concentrated to original volume by using an Amicon PM10 (Amicon Corp., Scientific Sys. Div., Lexington, Mass.), millipore-filtered (0.45-μm filter), and tested on B lymphocytes in the presence of 2 μg/ml TT.

Results

Effect of LMF-TT, NSF, and ASF on B Cells. LMF-TT and NSF eluted spontaneously after passage of LMF-TT over a TT immunosorbent column caused vigorous proliferation, IgG synthesis, and IgG anti-TT synthesis in autologous B cells (Table I and II). The addition of DT antigen to B-cell cultures containing LMF-TT or NSF resulted in anti-DT antibody synthesis (Table I).

ASF isolated over a TT immunosorbent column caused no significant proliferation or IgG synthesis in autologous B cells (Table I). However, in the presence of added TT, ASF induced anti-TT antibody synthesis in autologous B cells (Table I and II). Addition of DT antigen to ASF did not result in anti-DT antibody synthesis by the B-cell cultures. When LMF-TT was fractionated over a DT immunosorbent column, the 3 M NaCNS eluate from that column did not induce antibody synthesis in B cells regardless of whether DT or TT were present in the B-cell cultures (Table II).

ASF isolated over an anti-TT immunosorbent column showed properties similar to those of ASF isolated over a TT immunosorbent column (Table II). However, ASF isolated in this manner did not require the addition of TT to express its activity on B-cell cultures because the 3 M NaCNS eluate from the anti-TT immunosorbent column contained most of the TT antigen originally present in the T-cell supernates. When LMF-TT was passed over an anti-DT column, the 3 M NaCNS eluate from that column failed to induce specific antibody synthesis in autologous B cells (data not shown).

Effect of Anti-DRW Antisera to Ia-Like Antigens on ASF Activity. Adsorption of ASF over immunosorbent columns containing antisera to DRW antigens of the donor whose cells were used as a source of ASF, resulted in significant loss of ASF activity in eight of nine experiments using the B cells from six different donors. The exception shown in Table III, represents the failure of an immunosorbent containing an anti-

TABLE II

Effect of Antigen and Antibody Immunosorbent Columns on the Activity of LMF-TT on Autologous B-cell Cultures

Passage over immunosorbent	Eluate assayed	Antigen present in B-cell culture	IgG synthesis* Anti-TT	IgG synthesis* Anti-DT
			Δ(P-R) cpm ^{125}I	
Sepharose	Spontaneous	TT	+5,387	+127
	Spontaneous	TT, DT	+5,063	+1,036
Sepharose-TT	Spontaneous	TT	+6,687	+64
	Spontaneous	TT, DT	+5,750	+963
	3 M NaCNS	None	+312	+177
	3 M NaCNS	TT	+7,625	−109
	3 M NaCNS	DT	+421	+82
Sepharose DT	Spontaneous	TT	+5,512	−53
	3 M NaCNS	None	−71	+20
	3 M NaCNS	TT, DT	+46	+117
Sepharose anti-TT	Spontaneous	TT	+4,781	+216
	3 M NaCNS	TT, DT	+3,810	+39

* Results are expressed as cpm of ^{125}I-radiolabeled antigen precipitated by the supernatant fluid of 1×10^6 B cells.

DRW 5,6 antiserum to affect the activity of ASF derived from a DRW 4, DRW 5 donor (Table III). The loss of ASF activity that occurred after absorption with the relevant anti-DRW antisera ranged from 34 to 71% in eight experiments. Absorption of ASF with insolubilized antisera that did not recognize the DRW antigens of the ASF donor resulted in no significant loss of ASF activity (<11%) in five of five experiments using the cells of five different donors (Table III).

In eight of nine experiments using the B cells from six different donors, ASF activity was inhibited by the addition of antisera recognizing B-lymphocyte antigens on cultured B cells (Table III). The exception is again represented in Table III by the failure of the anti-DRW 5,6 antiserum to affect the response of B cells from DRW 4, DRW 5 donor to autologous ASF. In five of five experiments, the response of ASF of B cells from five different donors was not affected by the presence of anti-DRW antisera which did not recognize the DRW antigens present on the cultured B cells.

The activity of unfractionated LMF-TT and NSF was not inhibited by antisera to B-lymphocyte antigens and was not absorbed by such antisera regardless of whether the antisera recognized antigens present on the B cells of the ASF donor or not (data not shown).

Adsorption of ASF on Anti-IgM and Anti-IgG Immunosorbents. Passage of ASF over immunosorbent columns containing rabbit antisera specific to the heavy chain determinants of human IgG and IgM did not result in loss of ASF activity (Table IV).

Adsorption of ASF on Immunosorbents Containing Anti-Idiotypic Antiserum. Passage of ASF over immunosorbent columns containing the Ig fraction of an idiotypic antiserum to F $(ab')_2$ anti-TT derived from the ASF donor resulted in an almost complete loss of ASF activity (Table IV). Passage of ASF over immunosorbent columns containing normal rabbit IgG or the IgG fraction of an idiotypic antiserum to F $(ab')_2$ anti-TT

TABLE III

*Effect of Antisera to B-Cell Alloantigens on ASF Activity**

B-cell alloantigen of donor	Passage over immuno-sorbent	Alloantisera present in B-cell cultures	Anti-TT synthesis‡ Δ(P-R) cpm ^{125}I	Percent inhibition
DRW3 + DRW5	—	—	5,080	—
	Anti-DRW 4,5,6	—	2,489	52
	Anti-DRW 5,6	—	1,619	68
	Anti-DRW 6,7	—	4,635	9
	—	Anti-DRW 4,5,6	2,269	58
	—	Anti-DRW 5,6	2,030	60
	—	Anti-DRW 6,7	4,466	14
DRW4 + DRW5	—	—	3,515	—
	Anti-DRW 4,5	—	2,163	39
	Anti-DRW 5,6	—	3,415	2
	—	Anti-DRW 4,5	1,840	48
	—	Anti-DRW 5,6	2,978	15
DRW4 + DRW7	—	—	5,233	—
	Anti-DRW 4,5	—	3,216	38
	Anti-DRW 5,6	—	5,110	2
	—	Anti-DRW 4,5	2,964	43
	—	Anti-DRW 5,6	4,837	8
DRW3 + DRW7	—	—	4,612	—
	Anti-DRW 6,7	—	2,108	54
	Anti-DRW 4,5	—	4,465	3
	—	Anti-DRW 6,7	1,882	59
	—	Anti-DRW 4,5	4,071	12

* ASF was assayed on autologous B cells.

‡ counts per minute ^{125}I-TT precipitated by the supernate of 1 × 10^6 B cells.

from a donor different than the ASF donor did not result in loss of ASF activity.

Susceptibility of ASF to Enzymes. The proteolytic enzymes ficin and trypsin completely destroyed ASF activity. ASF was resistant to treatment with DNAase, RNAase, and amylase (Table V).

Gel Filtration of ASF on Sephadex G-100. After gel filtration over Sephadex G-100, ASF activity was recovered in fractions where proteins of mol wt of 25,000–75,000 are eluted (Table VI).

Discussion

The data presented in this paper demonstrate that supernates of TT-stimulated human T cells contain, in addition to the previously described NSF, an ASF capable of inducing anti-TT antibody synthesis in autologous B lymphocytes. The presence of both NSF and ASF together in supernates of antigen-stimulated human T cells is similar to the findings of Marrack and Kappler (15) in the murine system. ASF differs in several respects from NSF. NSF behaves like a polyclonal B-cell activator in that it triggers as much as 50% of autologous as well as allogeneic cells into proliferation and IgG synthesis and can induce specific antibody synthesis to a variety of antigens

TABLE IV

Absorption of ASF over Immunosorbent Columns Containing Anti-μ Chain, Anti-γ Chain, and Anti-Idiotypic Antisera

Experiment	Passage over immunosorbent	IgG anti-TT synthesis* Δ(P-R) ^{125}I cpm	Percent inhibition
1	None	+7,099	—
	Anti-IgG	+5,800	18
	Anti-IgM	+7,254	−2
	Anti-HSA	+6,914	3
2	None	+5,050	—
	Rabbit IgG	+4,083	19
	Rabbit anti-idiotypic IgG directed against‡ F (ab)$_2$ anti-TT of ASF donor	+579	89
	Rabbit anti-idiotypic IgG directed against§ F (ab)$_2$ anti-TT of unrelated donor	+3,865	23

* cpm of ^{125}I-TT precipitated by the supernatant fluid of 1 × 10^6 B cells.

‡ The IgG from this antiserum at a concentration of 10 mg/ml had a binding capacity of 950 ng of immunizing F (ab)$_2$ anti-TT per milliliter as calculated from the 50% binding point of a radioimmunoassay utilizing 20 ng of radiolabeled F (ab)$_2$ anti-TT and decreasing amounts of antiserum starting with 100 μl. Precipitates were made by using normal rabbit IgG and goat anti-rabbit IgG at equivalence. 100 ml of this anti-idiotypic IgG inhibited by more than 95% the binding of radiolabeled TT to 2 μg of IgG derived from the donor of the immunizing F (ab)$_2$ anti-TT. This assay was carried by adding different amounts of anti-idiotypic IgG to 2 μg of human IgG and 10 ng of radiolabeled TT. These latter amounts were chosen because they resulted in binding of 70% of the labeled TT to the human IgG which was precipitated using a myeloma IgG and rabbit anti-human IgG at equivalence. Anti-idiotypic IgG was coupled to an equal volume of packed Sepharose 4 B beads.

§ The IgG from this antiserum at a 10-mg/ml concentration had binding capacity of 1,330 ng of immunizing F (ab)$_2$ anti-TT per milliliter. 100 nl of this antiserum inhibited by 90% the binding of radiolabeled TT to IgG derived from the donor of the immunizing F (ab)$_2$ anti-TT. 100 nl of this anti-idiotypic IgG completely failed to bind to any of 20 ng of ^{125}I-radiolabeled (Fab)$_2$ anti-TT derived from the ASF donor and completely failed to inhibit the binding of radiolabeled TT to IgG derived from the ASF donor. All assays and the binding to Sepharose 4B were done as described in preceding paragraph.

TABLE V

Effect of Enzyme Treatment on ASF Activity

Treatment	Anti-anti-TT* Δ(P-R)cpm ^{125}I	Percent inhibition
None	7,566	—
DNase	9,074	19
RNase	6,004	21
Amylase	5,480	28
Ficin	470	94
Trypsin	531	93

* cpm of ^{125}I-TT precipitated by the supernatant fluid of 1 × 10^6 B cells.

TABLE VI

Estimate of Molecular Weight of ASF by Gel Filtration on Sephadex G-100

	IgG anti-TT synthesis* Δ(P-R) cpm ^{125}I
Unfractionated ASF	7,099
Sephadex G-100 eluants§	
Fraction I	68
Fraction II	5,711
Fraction III	5,794
Fraction IV	619
Fraction V	178

* cpm of ^{125}I-TT precipitated by the supernatant fluid of 1×10^6 B cells.

‡ The range of the molecular weights is: fraction I, >75,000; fraction II, 50,000-75,000; fraction III, 25,000-50,000; fraction IV, 12,500-25,000; fraction V, <12,500. The column was calibrated with human gammaglobulin (150,000), bovine serum albumin (68,000), ovalbumin (43,000), bovine pancreas chymotrypsinogen A (25,000), and horse heart cytochrome *c* (12,500). All fractions were concentrated to a volume equal to the original volume of ASF applied to the column.

added to the B-cell culture (7). Whereas, ASF triggers B cells into specific antibody synthesis, it can induce antibody synthesis only in the presence of the antigen used to stimulate helper factor production T cells (Tables I and II).

ASF was isolated from unfractionated T-cell supernates by adsorption onto immunosorbent columns containing cross-linked TT antigen. The material that spontaneously eluted from such columns was previously shown to contain NSF. The material that bound to the column and was eluted with 3 M NaCNS demonstrated ASF activity. ASF exhibited affinity exclusively to the TT antigen, which was used to stimulate helper factor production, but not to unrelated antigens such as DT. It seems that the affinity of ASF to cross-linked antigen is significantly higher than its affinity to soluble antigen because soluble TT antigen was present in the unfractionated T-cell supernates (LMF-TT). Furthermore, because soluble TT was shown not to stick nonspecifically to the column, as demonstrated using trace amounts of radiolabeled TT, it is unlikely that complexes of ASF and soluble TT (or TT fragments) absorbed nonspecifically to the column. Therefore, ASF must have preferentially bound to insolubilized TT in the presence of soluble TT antigen. This higher affinity of ASF to insolubilized antigen is in full agreement with the data of Munro et al. (16) on murine ASF induced by the synthetic antigen (T, G)-A--L.

ASF could also be isolated from supernates of TT-stimulated human T cells by passage over an immunosorbent column containing rabbit anti-TT antibody. It is likely that under these conditions soluble TT contained in the T-cell supernates bound to the insolubilized rabbit anti-TT and the resulting immobilized TT antigen retained ASF. Again, this retention was antigen specific in that ASF activity was not retained on immunosorbent columns containing antibody to DT.

ASF activity was expressed only in the presence of the antigen TT that was used to generate ASF. Thus, the material that bound to insolubilized TT caused autologous B cells to synthesize specific anti-TT antibody only if TT antigen was added concomitantly to the B-cell cultures. Addition of an unrelated antigen such as DT to these cultures in the presence of ASF did not result in anti-DT antibody synthesis,

demonstrating that ASF activity is exquisitely antigen specific. The antigen specificity of human ASF is in full agreement with the data on murine ASF (5, 15, 16).

ASF caused negligible proliferation and IgG production in B cells. This is in contrast to the NSF, previously shown to be present in the same unfractionated T-cell supernates, and which induced proliferation in as much as 50% of the B cells accompanied by vigorous IgG synthesis, only a small fraction of which had specificity to the TT antigen (7). It is probable that ASF triggers only those B cells that recognize TT antigen and that TT antigen may serve to focus ASF on the TT-specific B cells by binding to TT-specific surface immunoglobulin receptors. Because TT-specific B cells constitute a small fraction of the total number of B cells, triggering of the TT-binding B cells by ASF is therefore not expected to result in detectable proliferation or vigorous IgG synthesis.

The antigen-binding moiety in ASF is not a classical immunoglobulin molecule because ASF was not retained on columns containing antisera to the heavy chain determinants of human IgG and IgM. However, ASF was retained on columns containing anti-idiotypic antiserum raised in rabbits against F $(ab')_2$ anti-TT derived from the ASF donor. ASF activity was not diminished after passage over columns containing anti-idiotypic antiserum raised against F $(ab')_2$ anti-TT from an unrelated donor shown not to possess cross-reactive idiotypic determinants with the ASF donor. This data suggests that ASF shares antigen-binding idiotypic determinants with circulating antibody molecules. Because the T cell is the source of ASF and because shared antigen-binding idiotypic determinants has been identified on the surface of both B and T cells in experimental animals (17) it is likely that ASF contains the T-cell receptor for antigen. This data is in agreement with recent data obtained by Mozes (18) demonstrating that murine ASF binds to insolubilized anti-idiotypic antibodies.

With one exception, insolubilized anti-DRW antisera (antisera to Ia analogues) of the ASF donor partially removed ASF activity, suggesting that ASF contains Ia-like B-lymphocyte alloantigens. The exception represents the failure of an antiserum that recognizes DRW 5 and DRW 6 antigens to absorb ASF derived from a donor possessing the DRW 4 and DRW 5 antigens (Table III). Also, when this antiserum was added to cultures of B cells from the same donor, it failed to inhibit the B-cell response to autologous ASF. This failure cannot be explained by the antiserum being a weak one because the same antiserum absorbed substantially ASF derived from a second donor possessing the DRW 3 and DRW 5 antigens (Table III). It is possible, however, that much greater quantities of ASF were present in the T-cell supernates of the first donor exceeding the absorption capacity of the anti-DRW 5,6 antiserum. A more attractive, but equally speculative, possibility is that the antigenic determinants present in ASF may not be DRW antigens but the product of a gene closely linked to DRW 5 and absent in the donor possessing the DRW 4, DRW 5 antigens whose ASF activity was not affected by absorption with the anti-DRW 5,6 antiserum.

Similarly, and with the same exception discussed above, anti-DRW antisera inhibited the B-cell response to ASF when they were added to the B-cell culture provided they recognized DRW antigens present on the B cells. In contrast, the B-cell response to NSF was not inhibited by the addition of anti-DRW antisera that recognized the B-cell DRW antigens indicating that these antisera were not irreversibly inactivating, or killing, B cells under the present culture conditions.

In all experiments, anti-DRW antisera that did not recognize DRW antigens on the ASF donor failed to either absorb ASF or interfere in culture with the B-cell response to ASF.

Because ASF is the product of antigen-activated T cells, the source of Ia-like antigens in ASF may be the activated T cell itself, which has been demonstrated by David et al. (19) to express Ia antigens, or the Ia-bearing macrophage which is necessary for the T-cell activation by antigen. The present experiments do not allow a distinction between these two possibilities.

Whereas it has been clearly demonstrated in mice that the Ir-region determinants present on ASF are coded for the I-A subregion, no such analysis is at present feasible in man. Tissue typing for human B-lymphocyte antigens is still far from being exhaustive and the human alloantisera available are not sufficiently monospecific and do not distinguish between the putative determinants coded for by subregions of an Ir-like region of the human major histocompatibility complex. The observation that ASF from a single donor was inhibited by one but not the other of two antisera that recognized the DRW 5 antigen on the B cells of that donor suggests that the antigenic determinants present in ASF may not be DRW 5 but the product of a gene closely linked to DRW 5 and recognized by only one of the two antisera tested. Evidence that human B-cell alloantigens are coded for by more than one locus has been recently presented by several groups (20–22). Identification of which subregion of the Ir-like region in man is represented in ASF should be possible whenever antisera capable of distinguishing the products of the human Ir-like subregion genes become available.

Summary

Supernates of tetanus toxoid (TT) antigen-stimulated human T cells were studied for the presence of an antigen-specific T-cell helper factor (ASF). Supernates were circulated over an immunosorbent column consisting of insolubilized TT antigen. The material which bound to the column was eluted with 3 M NaCNS and was shown to contain a factor which in the presence of TT-induced specific IgG anti-TT antibody synthesis in autologous B cells without causing readily detectable proliferation. ASF activity was partially inhibited by antisera directed against the B-cell alloantigens of the ASF donor. Immunosorbent columns containing such antisera removed ASF activity. Immunosorbent columns containing antisera to human immunoglobulin heavy chain determinants did not remove ASF activity; whereas immunosorbent columns containing rabbit idiotypic antiserum directed against anti-TT antibodies completely removed ASF activity. ASF was destroyed by treatment with proteolytic enzymes; its molecular weight was estimated by Sephadex G-100 gel column chromatography to be between 25,000 and 75,000 daltons.

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