IN VITRO ANALYSIS OF ALLOGENEIC LYMPHOCYTE INTERACTION

VII. *I-A*-Restricted Self-reactive and Alloreactive Helper Components of Allogeneic Effect Factor are Distinct Donor T Cell-derived Ia^- Molecules that Recognize Ia Determinants on Antigen-presenting Cells*

By TERRY L. DELOVITCH, KATIE KAUFMAN, AND REGINALD M. GORCZYNSKI

From the Banting and Best Department of Medical Research, and the Department of Medical Biophysics, University of Toronto, Ontario, Canada M5G 1L6

Previously, we isolated and characterized an *I-A*-restricted alloreactive T helper (T_H)[1] component from an allogeneic effect factor (AEF) produced across an *H-2* incompatibility using graft-versus-host reaction (GVHR)-activated responder T cells and irradiated stimulator spleen cells (1). This AEF component was purported to be a secreted form of a T cell Ia alloantigen receptor. In this report, we extended these studies by the characterization of the T_H components of an AEF produced across the same *H-2* incompatibility using GVHR-activated responder T cells and irradiated stimulator T cell-depleted spleen cells. The use of such a stimulator cell population reduced the chance that any detectable *I-A*-restricted AEF T_H components were products of stimulator T cells (2). We also introduced some modifications to our method of biochemical fractionation of AEF that enabled us to detect both self-reactive and alloreactive *I-A*-restricted T_H components. Comparative functional and molecular analyses of these two *I-A*-restricted components suggest that they are structurally distinct responder T cell-derived Ia^- molecules that recognize *I-A*-encoded determinants on antigen-presenting cells (APC) of the responder and stimulator haplotypes, respectively. The data are discussed in terms of their relevance to the molecular basis of T cell alloreactivity.

Materials and Methods

Mice. Mice used were bred and maintained at the University of Toronto.

Antibodies. Ascites fluids of the rat AF.3.44.4 (330 μg IgM/ml) and F 30.60 (16 mg IgG/ml) hybridomas produced upon immunization with a mouse keyhole limpet hemocyanin (KLH)-specific T cell helper factor (T_HF) and a KLH-specific T cell suppressor factor (T_SF) (3), respectively, were kindly supplied by Dr. R. James and Dr. M. Feldmann, Department of

* Supported by grants MT 5729 and MA 5440 from the Medical Research Council of Canada.

[1] *Abbreviations used in this paper*: AEF, allogeneic effect factor; APC, antigen-presenting cell; BSA, bovine serum albumin; C′, rabbit serum complement; 1-D and 2-D, one and two dimensional; DTT, dithiothreitol; GVHR, graft-versus-host-reaction; IEF, isoelectric focusing; IL-2, interleukin 2; KLH, keyhole limpet hemocyanin; MLR, mixed lymphocyte reaction; MSA, mouse serum albumin; PBS, phosphate-buffered saline; PFC, plaque-forming cell; pI, isoelectric point; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SRBC, sheep erythrocyte; T_H, T helper cell; T_HF, T cell helper factor; T_S, T suppressor cell, T_SF, T cell suppressor factor; TPCK, L(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/83/06/1794/14 $1.00
Volume 157 June 1983 1794–1807

Zoology, University College, London, England. Affinity-purified $F(ab')_2$ of rabbit anti-rat Fab (3 mg IgG/ml) was a kind gift from Dr. M. Letarte, Division of Immunology, Hospital for Sick Children, Toronto, Ontario, Canada. Goat anti-rabbit IgG antiserum was purchased from Miles Laboratories Inc., Elkhart, IN. The HO.13-4 monoclonal anti-Thy-1.2 mouse IgM-secreting hybridoma (4) used to prepare T cell-depleted spleen cells was obtained from the Salk Institute Cell Distribution Center, La Jolla, CA. Mouse anti-Lyt-1.2 and anti-Lyt-2.2 monoclonal antibodies used to prepare B cell and APC suspensions were purchased from Cedarlane Laboratories, Hornby, Ontario.

Preparation of Cells. Suspensions of spleen cells that were either depleted of T cells (B + APC), or otherwise highly enriched for either B cells or APC, were prepared as described (5). The B cell and APC suspensions consisted of 1–3% Thy-1.2$^+$, ≤1% Ig$^+$ cells and 1–2% Thy-1.2$^+$, ≥95% Ig$^+$ cells, respectively (5). APC were irradiated with 3,000 rad before use (5).

Preparation, Fractionation, and Radioiodination of AEF. AEF was produced across an *H-2* incompatibility using GVHR-activated A.SW (*H-2^s*) responder T cells and irradiated (3,000 rad) A/WySn (*H-2^a*) stimulator spleen cells that were either depleted (i.e., treated with anti-Thy-1.2 plus complement [C']) or not depleted of T cells. Results obtained with AEF prepared with irradiated T cell-depleted stimulator spleen cells are presented. Two different batches of AEF were used in this study and each one yielded similar results. As was previously observed (1, 6), ≥90% of the activated responder T cell population were serologically shown to be of donor T cell origin. Thus, the use of such T cell-enriched responder and T cell-depleted stimulator populations enhanced the likelihood that alloactivated responder T cells are the source of AEF-derived helper components.

About 25 ml of unseparated serum-free AEF was concentrated to 5 ml and chromatographed at 4°C on Ultrogel ACA 54 (Fisher Scientific Co., Toronto, Ontario), equilibrated with sterile 0.9% NaCl (1, 5). Selected biologically active fractions (8 ml) were pooled, concentrated to 3 ml and further resolved by chromatofocusing at 4°C on PBE 94 gel (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) using 0.025 M imidazole-HCl, pH 7, as start buffer and Polybuffer 74-HCl (Pharmacia Fine Chemicals), pH 4.0, as eluent (5, 7). Active fractions (8 ml) found in the pH 5.5–6.0 range were pooled and refocused on PBE 94 using the same start buffer and Polybuffer 74-HCl, pH 5.0, as eluent. Chromatofocused fractions were neutralized to pH 7.2, with 1 M Tris base, pH 11, dialyzed vs. 0.04 M NaCl, and concentrated 3–4-fold before assay. After fractionation, the yield of certain AEF components was estimated spectrofluorimetrically, as described (5).

About 250 ng (4 pmol) of the *I-A*-restricted AEF helper components obtained after the second step of chromatofocusing were radioacylated with ^{125}I using the Bolton-Hunter reagent (>1,375 Ci/mmol sp act; Amersham Corp., Oakville, Ontario) and then further processed before storage at −70°C as described (5).

Biochemical Analysis of AEF. Treatment with various enzymes (mercuripapain, neuraminidase, alkaline phosphatase, trypsin), immunoprecipitation with protein A-Sepharose CL 4B (Pharmacia Fine Chemicals), one-dimensional (1-D) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF) on 1-D slab gels, and peptide mapping on IEF tube gels of ^{125}I-labeled *I-A*-restricted helper components of AEF were performed exactly as described (5).

Biological Assay of AEF. After each step of biochemical fractionation of AEF, aliquots (70 μl) of isolated fractions (8 ml) were assayed in triplicate at either a 1:3, 1:6, or 1:9 final dilution for their capacity to activate an in vitro primary anti-sheep erythrocyte (SRBC) 5-d direct plaque-forming cell (PFC) response (1). SRBC from a single sheep (sheep No. 5, Woodlyn Laboratories, Guelph, Ontario) were used. Cultures (0.21 ml) in RPMI 1640 medium, supplemented with 5% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml gentamycin, contained 10^6 SRBC as antigen and either 10^6 T cell-depleted B + APC or 10^4 isolated APC and 5×10^5 isolated B cells.

Results

Fractionation of AEF T_H Components. Fig. 1*A* shows that fractions of AEF eluting from ACA 54 in eight different mol wt ranges, designated pools I–VIII, displayed T_H

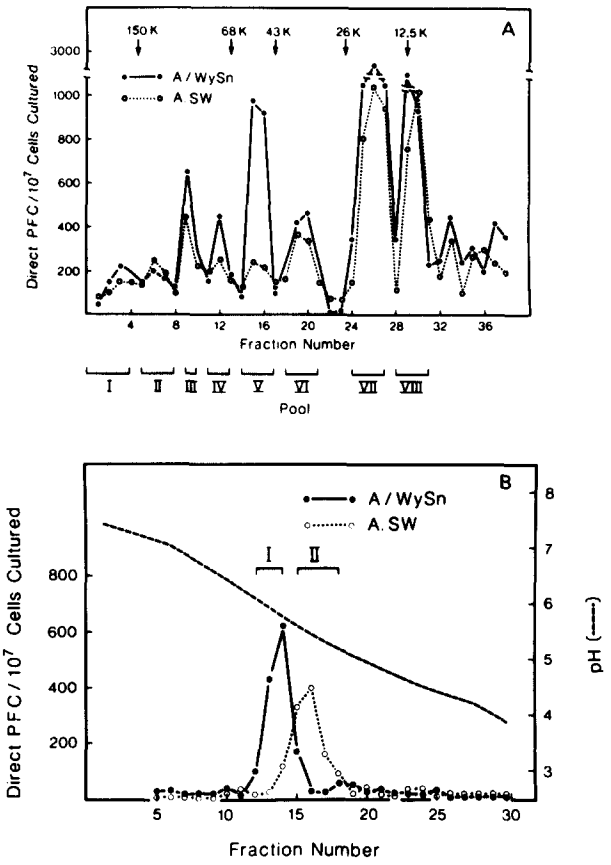

FIG. 1. (*A*) Assay of AEF activity after gel filtration on ACA 54. About 25 ml of AEF was concentrated to 5 ml and chromatographed on ACA 54. Column fractions (8 ml) were collected and assayed at 1:9 dilution in a primary in vitro anti-SRBC direct PFC response of either A/WySn (●) or A.SW (○) T cell-depleted spleen cells (10^6). The positions of the molecular weight markers rabbit IgG (150,000), bovine serum albumin (86,000), ovalbumin (43,000), α-chymotrypsinogen (26,000) and cytochrome C (12,500) are indicated by arrows. The peaks of T_H activity observed are designated as pools I–VIII. (*B*) Assay of AEF activity after the first stage of chromatofocusing. The *I-A*-restricted ACA 54 pool V in (*A*) was concentrated and chromatofocused on PBE 94 using Polybuffer 74-HCl, pH 4.0, as eluent. Fractions (8 ml) were neutralized, dialysed, concentrated, and assayed at a 1:6 dilution for their T_H activity with either 10^6 A/WySn (●) or A.SW (○) T cell-depleted spleen cells. The peaks of T_H activity are designated as pools I and II.

activity for T cell-depleted spleen cells of both the stimulator and responder haplotypes. By contrast to our previous identification of only two major pools of AEF T_H activity upon assay of the fraction of responding microcultures (1), quantitation of direct PFC in the in vitro primary anti-SRBC responses illustrated here enabled us to detect several distinct pools of AEF T_H activity. Interestingly, an *H-2*-restricted T_H activity for A/WySn lymphocytes (i.e., alloreactivity) was found to be associated only with pool V, which contained components in the 50,000–70,000 mol wt range. A strain distribution analysis similar to that previously described (7) showed this T_H activity to be $I\text{-}A^k$ restricted. Little *H-2*-restricted T_H activity for A.SW lymphocytes (i.e., self-reactivity) was evident at this stage of AEF fractionation.

Since ACA 54 pool V of Fig. 1 *A* contained strong T_H alloreactivity and very much

weaker T_H self-reactivity, it was of interest to determine whether these two reactivities are mediated by the same or different components in this pool. Pool V was therefore further fractionated by chromatofocusing. Fig. 1 *B* shows a chromatofocusing pattern of ACA 54 pool V obtained using a descending pH gradient of 7.4–3.8. The T_H activity observed appeared in the pH 5.4–6.0 range and fractions eluting in this range differed from each other by about 0.2 pH units. A sharp predominant peak of T_H alloreactivity for A/WySn T cell-depleted spleen cells focused at an isoelectric point (pI) of ~5.8 (pool I), while a broader and lesser peak of T_H self-reactivity for A.SW T cell-depleted spleen cells focused at pI 5.5 (pool II). These data suggested that alloreactive and self-reactive T_H components of AEF might be separable according to their difference in net molecular charge.

Resolution and Target Cell of Action of Alloreactive and Self-reactive AEF T_H Components. An attempt was made to improve the resolution of the putative alloreactive and self-reactive T_H components of AEF and to identify the target cell of action of these T_H components. Pools I and II of Fig. 1 *B* were rechromatofocused using a descending pH gradient of 7–5, which permitted the elution of fractions that differed by <0.1 pH units. The eluted fractions were assayed for their T_H activity in the presence of either syngeneic or allogeneic mixtures of isolated B10.A (*H-2*^{a}; histocompatible with stimulator strain (A/WySn) and B10.S (*H-2*^{s}; histocompatible with responder strain A.SW) splenic APC and B cells.

Fig. 2 illustrates that alloreactive and self-reactive T_H components of AEF were well resolved by this second step of chromatofocusing. The alloreactive component of about pI 5.8 was active in the presence of B10.A APC and either syngeneic B10.A or allogeneic B10.S B cells. The self-reactive component of about pI 5.5 was active in the presence of B10.S APC and either syngeneic B10.S or allogeneic B10.A B cells. The self-reactive component likely recognizes *I-A*^{s}-encoded alloantigens on B10.S APC since no *I-E* alloantigens are expressed by the *H-2*^{s} haplotype. For simplicity, these alloreactive pI 5.8 (Fig. 2, fractions 16–17) and self-reactive pI 5.5 (fractions 20–21) AEF components will be designated as T_H^{IA}-1 and T_H^{IA}-2, respectively. The data indicate that T_H^{IA}-1 and T_H^{IA}-2 differ in their pI value and that their *I-A*-restricted activity is operative at the level of recognition of Ia molecules on an APC and not a B cell.

Partial strain distribution analyses of the activity of T_H^{IA}-1 and T_H^{IA}-2 have shown that APC of strains that express *I-A*^{k} or *I-A*^{d} determinants are required to detect T_H^{IA}-1 activity, whereas APC of strains that express *I-A*^{s} or *I-A*^{d} determinants are required to detect T_H^{IA}-2 activity (data not shown). No responses were obtained for either AEF T_H component in the presence of APC of strains that express *I-A*^{b} or *I-A*^{f} determinants. The patterns of reactivity of T_H^{IA}-1 and T_H^{IA}-2 are most likely attributable to structural homologies that exist between *I-A*-encoded Ia antigens of different haplotypes (8). Although both T_H^{IA}-1 and T_H^{IA}-2 display alloreactivity, only T_H^{IA}-2 exhibits self-reactivity for the responder haplotype APC (Fig. 2). We shall therefore continue to refer to T_H^{IA}-2 as a self-reactive component. T_H^{IA}-1 and T_H^{IA}-2 were active only if added during the first 24 h of culture. A T_H/APC interaction in the presence of antigen must therefore preempt B cell activation, differentiation, and Ig secretion. These observations are compatible with our tentative conclusions reached earlier using a less well purified ACA 54 column-fractionated *I-A*-restricted T_H component of AEF (9).

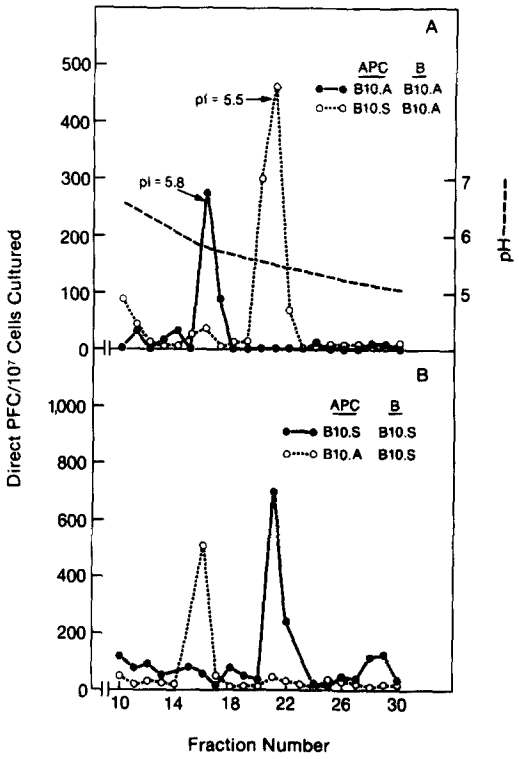

FIG. 2. Assay of AEF activity after the second stage of chromatofocusing. Pools I and II of Fig. 1 *A* were each concentrated and rechromatofocused on PBE 94 using Polybuffer 74-HCl, pH 5.0, as eluent. Fractions (8 ml) were tested at a 1:3 dilution for their T_H activity with 5×10^5 B cells of either B10.A (*A*) or B10.S (*B*) origin and APC (10^4) from each strain as shown.

T_H^{IA}-1 and T_H^{IA}-2 are of limited heterogeneity in that each focused rather sharply, with their predominant activity being present in only two fractions (Fig. 2). Similar chromatofocusing patterns were previously obtained for two *I-J*-restricted AEF T_H components (5). Purified homogeneous proteins such as IgG H and L chains, hemoglobin, and ovalbumin also focus under similar conditions in three fractions. To estimate the degree of purification achieved for T_H^{IA}-1 and T_H^{IA}-2, we used a method of analysis of total activity initially devised for interleukin 2 (IL-2) (10) that was subsequently adapted for AEF (11). The yields of T_H^{IA}-1 and T_H^{IA}-2 at each stage of fractionation were monitored spectrofluorimetrically (8) and were used to calculate the specific activity of the various fractions. In this way, the extent of final purification of T_H^{IA}-1 was estimated to be about 900-fold (Table I). A similar result was obtained for T_H^{IA}-2 (not shown). This level of purification is comparable to the 800-fold purification reported for *I-J*-restricted AEF T_H components (5) and validates the reproducibility of this method.

Before fractionation, the 25 ml of AEF contained about 2.5 mg of total protein. The yields of T_H^{IA}-1 and T_H^{IA}-2 after the second step of chromatofocusing were ~2 and 1 μg, respectively. Since the greater proportion of the T cells ($\geq$90%) present in the cultures used to produce AEF were of donor origin (see Materials and Methods), we assume that both T_H^{IA}-1 and T_H^{IA}-2 are responder T cell derived. To detect T_H^{IA}-1 and

TABLE I
Purification of T^{IA}_H-1

Stage of fractionation	Protein	Total activity*	Specific activity‡	Purification fold
	mg			
Unseparated AEF	2.5	150	60	1
ACA 54	0.67	422	630	10.5
Chromatofocusing-1	0.014	352	25,200	420
Chromatofocusing-2	0.002	107	53,460	891

* One unit of AEF activity was assigned to the amount present in 1 ml of a 1:10 dilution of unseparated AEF. This dilution produced a response of ~500 direct PFC/10^7 cultured A/WySn T cell-depleted spleen cells and was used to compute the potency relative to the standard of unseparated AEF. The total activity of different fractions assayed by serial dilution was calculated at various stages of purification by multiplying the fraction volume by its potency relative to unseparated AEF.
‡ Specific activity = total activity/protein.

T^{IA}_H-2 activities, it is essential that alloactivated nonirradiated dividing responder T cell blasts be used for the production of AEF. Supernatants harvested after 16 h of coculture of nonactivated responder thymocytes and irradiated stimulator normal spleen cells did not elicit any *I-A*-restricted T_H activity (unpublished data). This lends further support to the notion that T^{IA}_H-1 and T^{IA}_H-2 are responder T cell-derived. Insofar as 2.5 × 10^8 activated responder T cells are required to generate 25 ml of AEF, it follows that on the order of 1 μg of T^{IA}_H-1 and T^{IA}_H-2 are each secreted and/or shed from 10^8 alloactivated T cells. These yields are maximum estimates since, although T^{IA}_H-1 and T^{IA}_H-2 were purified about 900-fold, they were presumably not purified to homogeneity. Nonetheless, the yields obtained concur with a yield of ~0.5 μg/10^8 cells expected for a eukaryotic plasma membrane-associated glycoprotein. These results also imply that ~0.01% of total protein synthesis in an activated T cell may be devoted to the synthesis of T^{IA}_H-1 and T^{IA}_H-2. The accuracy of this estimate depends on whether these proteins may also be found in greater amount in a membrane-associated form and/or cytoplasmic form. Based on a 68,000 mol wt for T^{IA}_H-1 and T^{IA}_H-2 (see Fig. 3), we deduce that these proteins are biologically active at the level of 1 pmol (i.e., 80 pg) per culture of 10^4 APC and 5 × 10^5 B cells. This picomolar concentration range of activity is very similar to that previously found for *I-J*-restricted AEF T_H components (5), IL-1 (11), and IL-2 (12).

Biochemical and Serological Analysis of T^{IA}_H-1 and T^{IA}_H-2. Due to the existing intrigue concerning the functional and molecular interrelationship between T cell alloreactivity and self-reactivity, we wished to compare the structural properties of the alloreactive T^{IA}_H-1 and self-reactive T^{IA}_H-2 components. T^{IA}_H-1 and T^{IA}_H-2 were characterized with respect to their size, subunit structure, sensitivity to proteolysis, charge heterogeneity, tryptic peptide map, and expression of certain serological determinants.

Aliquots of chromatofocused T^{IA}_H-1 and T^{IA}_H-2 (see Fig. 2) were radioiodinated and analyzed by 1-D 10% SDS-PAGE under reducing conditions before and after digestion with papain (11, 13). Before digestion, T^{IA}_H-1 (Fig. 3*A*) and T^{IA}_H-2 (Fig. 3*B*) each migrated as a single polypeptide chain of about 68,000 mol wt; identical results were obtained during electrophoresis under reducing conditions (data not shown), as

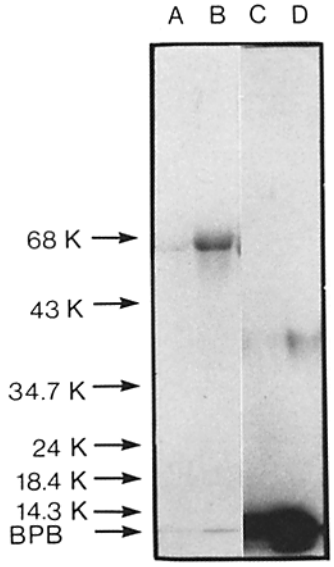

FIG. 3. SDS-PAGE of ^{125}I-labeled T_H^{IA}-1 and T_H^{IA}-2. Aliquots of chromatofocused T_H^{IA}-1 (lanes A and C) and T_H^{IA}-2 (lanes B and D) of Fig. 2 were radioiodinated, lyophilized, resuspended, and then incubated for 1 h at 37°C with 5 μg/ml of mercuripapain (enzyme/protein = 1:100) in 20 μl of PBS containing 1 mM dithiothreitol and 2.5% glycerol. Control samples were incubated in the absence of enzyme. Samples were electrophoresed before (lanes A and B) and after (lanes C and D) enzymatic digestion under nonreducing conditions on a 10 × 14 cm × 0.8 mm polyacrylamide (10% wt/vol) slab gel for 3½ h at 20 mA. About 31,000 and 58,000 trichloroacetic acid-precipitable counts per min were loaded in lanes A and B, respectively, and ~16,000 and 29,000 trichloroacetic acid-precipitable counts per min were loaded in lanes C and D, respectively. The gel was dried and exposed for 4 d to Kodak XRP-1 film using a Cronex Lightning Plus image-intensifying screen. The position of migration of the molecular weight markers for bovine serum albumin (68 K), ovalbumin (43 K), pepsin (34.7 K), trypsinogen (24 K), β-lactoglobulin (18.4 K), lysozyme (14.3 K), and bromophenol blue dye (BPB) are indicated by arrows. The same result was obtained after a 14-d exposure of the gel.

previously reported (1). T_H^{IA}-1 and T_H^{IA}-2 therefore do not appear to possess any subunit structure. Papain cleaved T_H^{IA}-1 (Fig. 3*C*) and T_H^{IA}-2 (Fig. 3*D*) into a single major band that appeared at 40,000. It is uncertain whether an additional fragment migrated in the 14,000 region at or immediately above the bromophenol blue marker dye. Several smaller ^{125}I-labeled peptides as well as residual free ^{125}I could also electrophorese in this region. A higher percentage acrylamide slab gel (e.g., 15% wt/vol) may be necessary to resolve this uncertainty. Thus, these AEF T_H components are cleaved by papain into a 40,000 mol wt fragment and perhaps lower mol wt peptides. Several small peptides but no larger fragments are obtained by digestion of T_H^{IA}-1 and T_H^{IA}-2 with other proteases such as pepsin and trypsin.

Since T_H^{IA}-1 and T_H^{IA}-2 are proteins of 68,000 mol wt that differ by only 0.3 pI U in their net charge, we checked whether their charge difference could be accounted for by either altered amounts of glycosylation and/or phosphorylation. ^{125}I-labeled T_H^{IA}-1 and T_H^{IA}-2 were analyzed by IEF (11) before and after incubation with either neuraminidase or alkaline phosphatase. Clusters of about five major IEF bands were observed for both undigested T_H^{IA}-1 and T_H^{IA}-2 (data not shown). The T_H^{IA}-1 cluster appeared in the pI 5.7–5.9 range and the T_H^{IA}-2 cluster in the pI 5.4–5.7 range. These pI values are consistent with the pI 5.8 and pI 5.5 values estimated by chromatofo-

cusing of T_H^{IA}-1 and T_H^{IA}-2, respectively. Treatment of T_H^{IA}-1 and T_H^{IA}-2 with either neuraminidase or alkaline phosphatase did not cause any charge shifts in their IEF patterns. It is unlikely, therefore, that the charge difference between T_H^{IA}-1 and T_H^{IA}-2 is attributable to a posttranslationally altered amount of sialylation or phosphorylation of these two proteins. However, the possibility that the multiplicity of IEF bands observed for these proteins results from microheterogeneities introduced during radioacylation (14) and/or from alternate forms of glycosylation cannot be ruled out.

To determine whether any of the charge heterogeneity between T_H^{IA}-1 and T_H^{IA}-2 results from a difference in their amino acid sequence, these proteins were subjected to a method of peptide-mapping analysis designed for picomolar quantities of radioiodinated protein (14). ^{125}I-labeled reduced and alkylated T_H^{IA}-1 and T_H^{IA}-2 were rechromatofocused to remove minor contaminants such as IL-2 and were then digested to completion with trypsin (5). Under these conditions, protein acylation results in derivatized lysine residues that are resistant to trypsinization, and proteolysis occurs only at arginine and alkylated cysteine residues. The sensitivity and reproducibility of this peptide-mapping technique was previously checked by comparative analyses of the maps of mouse serum albumin (MSA) and bovine serum albumin (BSA) and of alloreactive and self-reactive *I-J*-restricted AEF T_H components (5).

^{125}I-labeled tryptic peptides were separated on IEF gels using a pH gradient of 4–9.7 (Fig. 4). Nine peptides of T_H^{IA}-1 and eight peptides of T_H^{IA}-2 were observed. Seven of nine peptides (~80%) are shared between T_H^{IA}-1 and T_H^{IA}-2. T_H^{IA}-1 contains

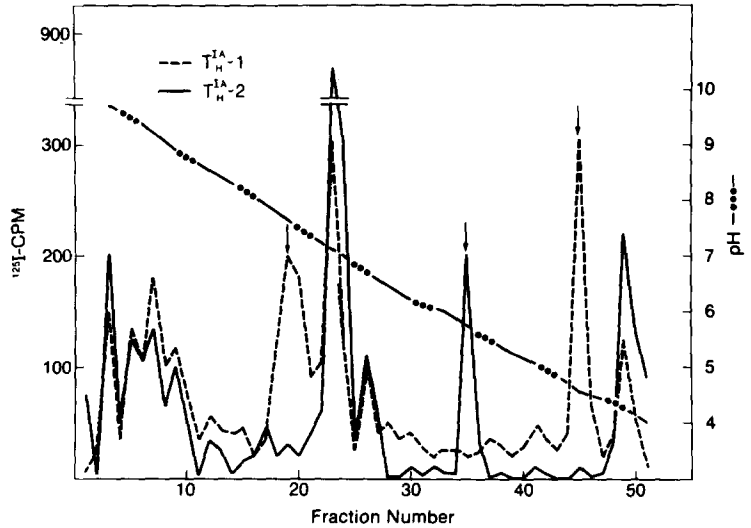

FIG. 4. Tryptic peptide maps of ^{125}I-labeled T_H^{IA}-1 and T_H^{IA}-2. Aliquots (250 ng) of ^{125}I-labeled T_H^{IA}-1 and T_H^{IA}-2 were reductively methylated, alkylated, and digested with 10 μg of L(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone (TPCK) trypsin in 0.1 M NH_4HCO_3 for 3 h at 37°C, using 500 μg of ovalbumin as carrier. An additional 10 μg of TPCK-trypsin was then added and the incubation continued for 15 h at 37°C. Samples were lyophilized, resuspended, and focused on 11 × 0.6 cm tube gels for 16 h at 400 V in the pH range of 4–10. Gel slices (1 mm) were cut and the radioactivity determined in a γ-counter. A background level of radioactivity (40 cpm) was subtracted from all samples. The pH of 2-mm gel slices of a tube gel run simultaneously in parallel is shown. To align the peptide maps, the pH of the peak fractions of each peptide detected was measured. T_H^{IA}-1 and T_H^{IA}-2 peptides that differ from each other are designated by arrows.

TABLE II

Serological Analysis of T^{IA}_H-1 and T^{IA}_H-2

Antibody	CPM precipitated*	
	T^{IA}_H-1	T^{IA}_H-2
AF.3.44.4 (anti-T_HF)	492	423
F 30.60 (anti-T_SF)	485	402
Normal rat serum‡	347	396
None‡	228	258

* To 80 μl of PBS containing 50,000 cpm of either T^{IA}_H-1 or T^{IA}_H-2 was added either 20 μl of anti-T_HF ascites (330 μg IgM/ml) or 1 μl of anti-T_SF ascites (16 mg IgG/ml). Reaction mixtures were incubated overnight at 4°C, 20 μl of affinity-purified F(ab')$_2$ of rabbit anti-rat Fab (3 mg IgG/ml) added, and the incubation extended for 8 h at 4°C. Subsequently, 100 μl of a goat anti-rabbit IgG was added to the mixtures. Reactions were terminated after an additional 16 h at 4°C by centrifugation at 6,300 *g* for 5 min. Immunoprecipitates were washed five times with 1 ml of PBS containing 0.5% Nonidet P-40, 0.1% SDS, 5 mM KI, and 0.5 mM phenylmethylsulfonyl fluoride, air dried, and their radioactivity determined in a γ-counter.

‡ Control samples were either reacted with 5 μl of Lewis normal rat serum instead of the rat monoclonal antibodies, or otherwise treated with only the second and third step reagents.

two peptides absent from T^{IA}_H-2, and T^{IA}_H-2 contains one peptide absent from T^{IA}_H-1. Thus, it seems that T^{IA}_H-1 and T^{IA}_H-2 are homologous but nonidentical proteins, and that a small number of amino acid interchanges in their primary structure might account for some of their net charge difference.

An earlier serological analysis (1) of an alloreactive, *I-A*-restricted, AEF T_H component demonstrated that it does not bear any Ia, V_H, C_H or L chain determinants. Similar results were obtained with the T^{IA}_H-1 and T^{IA}_H-2 isolated and characterized in this study. Other studies conducted with heteroantisera and monoclonal antibodies, produced against either T_H or T_S factors, have suggested by analogy to Ig molecules that factors bear isotypic determinants in their "constant region" (reviewed in reference 3). Accordingly, it was of interest to examine T^{IA}_H-1 and T^{IA}_H-2 for their expression of such putative isotypic determinants.

Aliquots of ^{125}I-labeled T^{IA}_H-1 and T^{IA}_H-2 were incubated with rat monoclonal antibodies reactive with either a KLH-specific T_HF or a KLH-specific T_S factor (T_SF) (3). Immunoprecipitates were formed using F(ab')$_2$ of rabbit anti-rat Fab and goat anti-rabbit IgG. Table II shows that no specific precipitation was obtained with anti-T_HF and anti-T_SF. T_SF does not share epitopes with T_HF (3) and would not be expected to share epitopes with T^{IA}_H-1 and T^{IA}_H-2. Although rat anti-T_HF does not react with T^{IA}_H-1 and T^{IA}_H-2, the possibility remains that T_HF, T^{IA}_H-1, and T^{IA}_H-2 express another as yet undetected epitope(s) in common.

Discussion

The *I-A*-restricted alloactivated responder T_H cell-derived AEF helper component that we previously characterized (1) was primarily alloreactive in its specificity but it also possessed self-reactivity, albeit considerable less. It is conceivable that this pattern of anti-Ia-like cross-reactivity was mediated by either a single T cell molecule or by two different T cell molecules each with a distinct specificity. We therefore attempted to increase the level of purification of this AEF alloreactive T_H component with the

aim (*a*) to determine if it were possible to biochemically resolve an alloreactive component from a self-reactive component; (*b*) to identify the target cell and mechanism of action of the alloreactive and self-reactive components; and (*c*) to gain more insight into the molecular basis of T cell alloreactivity and self-reactivity.

Using a method of fractionation of AEF that involved chromatography on ACA 54 and two stages of chromatofocusing, we separated an *I-A*^{k}-restricted alloreactive T_H component (T_H^{IA}-1) of 68,000 mol wt and pI 5.8 from an *I-A*^{s}-restricted self-reactive component T^{IA}-2 of 68,000 mol wt and pI 5.5 (Table III). The principle of this simple fractionation scheme is similar to that used in two-dimensional (2-D) gel electrophoresis, which separates proteins on the basis of molecular size and charge. Proteins such as Ia antigens that are resolved by 2-D gels may be highly purified, and when cut out of such gels, digested, and analyzed, yield a homogeneous tryptic peptide profile (15). The level of purification achieved here for T_H^{IA}-1 and T_H^{IA}-2 was estimated to be ~900-fold. Before peptide-mapping analyses, this amount of purification was presumably enhanced still further after a third chromatofocusing of T_H^{IA}-1 and T_H^{IA}-2 to remove contaminating IL-2. Elution of T_H^{IA}-1 and T_H^{IA}-2 activity from the 68,000 mol wt region of an SDS slab gel should enable us to conclude unequivocally that these helper activities are conferred by 68,000 mol wt proteins.

The biochemical fractionation scheme adopted here provided three additional advantages over the one previously used. First, chromatofocusing is more rapid, efficient, and easier to perform (5) than the horizontal flat bed and vertical column IEF techniques previously used (1). Second, the high degree of resolution achieved by chromatofocusing of proteins of very similar molecular charge permitted the separation of the T_H^{IA}-2 self-reactive component; this degree of resolution was not easily obtained with the methods of IEF used previously (1). Third, chromatofocusing eliminated the use of Ampholines (LKB Instruments, Inc., Rockville, MD) which invariably deplete AEF T_H activity, perhaps by denaturing T_H^{IA}-1 and T_H^{IA}-2 and lowering their relative affinity for their respective *I-A* determinants. Since T_H^{IA}-1 and T_H^{IA}-2 are generated by the allostimulation of responder T cells and since alloactivation both in vivo and in vitro are required to detect the activity of these proteins (reviewed in reference 5), the affinity of interaction between T_H^{IA}-1 and allo-Ia determinants may be higher than that between T_H^{IA}-2 and self-Ia determinants. Although T_H^{IA}-1 and T_H^{IA}-2 were isolated with approximately the same yield (i.e., ~1 $\mu g/10^8$ activated T cells) and are both active in the 1 pmol concentration range, the ability to detect their biological activity after various methods of fractionation presumably depends on their

TABLE III
Properties of T_H^{IA}-1 and T_H^{IA}-2

Property	T_H^{IA}-1	T_H^{IA}-2
Molecular weight	68,000	68,000
pI	5.8	5.5
Papain-cleavage fragments	40,000	40,000
Percent shared peptides	80	80
Cellular origin	Lyt-1$^+$2$^-$ T_H cell	Lyt-1$^+$2$^-$ T_H cell
Target cell	*I-A*^{k} APC	*I-A*^{s} APC
Recognition of *I-A* determinants	Allo-*I-A*^{k}	Self-*I-A*^{s}
Expression of Ia determinants	No	No

recovery with suitably high affinity for their target antigens.

Peptide-mapping analyses revealed that T_H^{IA}-1 and T_H^{IA}-2 share ~80% of their tryptic peptides. However, only a limited number of peptides (eight or nine) were observed for these proteins as a result of labeling them in their arginine and alkylated cysteine residues. Resolution of peptides derived by tryptic digestion of T_H^{IA}-1 and T_H^{IA}-2 labeled biosynthetically in their leucine and arginine residues might ultimately reveal more peptide heterogeneity between these two proteins. By analogy to tryptic peptide analyses of H-2K and H-2D proteins (16), an 80% degree of peptide sharing suggests that T_H^{IA}-1 and T_H^{IA}-2 are 90–95% homologous in their amino acid sequence. Since T_H^{IA}-1 and T_H^{IA}-2 were each purified probably greater than 900-fold before being analyzed by peptide mapping, it appears that T_H^{IA}-1 and T_H^{IA}-2 are homologous but nonidentical proteins. Further purification and characterization of T_H^{IA}-1 and T_H^{IA}-2 should confirm whether T_H^{IA}-1 and T_H^{IA}-2 are structurally different.

The *I-A*-restricted T_H^{IA}-1 and T_H^{IA}-2 polypeptides differ structurally from the previously described *I-J*-restricted self-reactive (T_H^{IJ}-1) and alloreactive (T_H^{IJ}-2) AEF T_H components produced across an *I-J* incompatibility (5). Whereas T_H^{IJ}-1 and T_H^{IJ}-2 share 80% of their tryptic peptides, these proteins share only 50–60% of their tryptic peptides with either T_H^{IA}-1 or T_H^{IA}-2. These four different proteins may therefore possess as much as 70–80% amino acid sequence homology. If they constitute the secreted forms of distinct T cell surface-associated Ia antigen receptors (1, 5), then T cell receptors for a given set of related antigens may be structurally quite homologous to each other. The suggested high degree of homology of self-Ia and allo-Ia T cell receptors is consistent with the hypothesis that T cell alloreactivity originates from cross-reactions of self-reactive T cells (17–21).

Papain, when incubated at 5 μg/ml for 1 h at 37°C with T_H^{IA}-1 and T_H^{IA}-2, splits these proteins into a single 40,000 mol wt fragment. A 20–30,000 mol wt fragment was not observed under these conditions or during digestion for 5–30 min at 37°C with 1 μg/ml of the enzyme (data not shown). The latter conditions resulted in 40,000 mol wt fragments that constituted ~75% of the digested product. The papain digestion pattern of T_H^{IA}-1 and T_H^{IA}-2 indicates that these proteins may differ structurally from another putative alloactivated T cell receptor that is split by plasmin into 45,000 and 25,000 mol wt fragments (22) and from an antigen-specific T_S molecule that is cleaved by papain into 45,000 and 24,000 mol wt fragments (13). The 25,000 mol wt fragment of the alloactivated T cell molecule has antigen-binding capacity, although the nature of the antigen (H-2 or Ia molecules) is undefined (22). The T_S molecule 45,000 mol wt fragment specifically suppresses and lacks antigen-binding activity while the T_S molecule 24,000 mol wt fragment does not suppress but has antigen-binding activity. By contrast, our preliminary data (not shown) indicate that the T_H^{IA}-1 and T_H^{IA}-2 40,000 mol wt fragments do not have any T_H activity and therefore probably do not retain their ability to recognize *I-A*-encoded determinants. Thus, antigen-specific T cell molecules may differ structurally from alloantigen-specific T cell molecules.

The cellular origin of T_H^{IA}-1 and T_H^{IA}-2 is most probably an Lyt-1^+2^- alloactivated responder T cell. This reasoning follows from the fact that these proteins are derived from mixed lymphocyte reaction (MLR) cultures of responder cells that were >90% GVHR-donor T cell in origin and from irradiated stimulator spleen cells that were depleted of T cells. GVHR- and MLR-activated T cell blasts that have T_H activity bear the Lyt-1^+2^- surface antigen phenotype (6). It shall prove interesting to determine

whether T_H^{IA}-1 and T_H^{IA}-2 are products of the same or, as expected, different activated T_H cells.

The notion that self-Ia and allo-Ia receptors appear on distinct activated T cells, and that such receptors differ structurally perhaps due to somatic mutation, is in concert with recent evidence that suggests that precursor T cells express anti-Ia receptors before their entry into the thymus (23). In an immunologically tolerant environment, precursor T cells may recognize allo-Ia determinants and be rendered nonfunctional. However, during an allogeneic stimulation in a nontolerant environment, such as that which occurs in a GVHR used in the first stage of AEF production, a subpopulation of donor thymocytes that express self-Ia (i.e., anti-donor Ia) receptors are probably stimulated in the irradiated host environment to express allo-Ia (i.e., anti-host Ia) receptors. Subpopulations of GVHR-activated T cells that express either self-Ia receptors or allo-Ia receptors can in fact be found (6). Future structural studies of products of clones of alloactivated *I-A*-restricted T_H cells should assist with the further elucidation of this problem.

Functional analyses of T_H^{IA}-1 and T_H^{IA}-2 demonstrated that T_H^{IA}-1 recognizes allo-*I-A*-encoded determinants on an APC of the stimulator haplotype and that T_H^{IA}-2 recognizes self-*I-A*-encoded determinants on an APC of the responder haplotype. The genotype of the APC and not the B cell proved important for T_H^{IA}-1 and T_H^{IA}-2 activity. These observations are compatible with those that indicate that the genotype of the APC and not the T cell dictates the specificity of an antigen-induced T cell proliferative response (21, 24). The data obtained herein with polyclonally alloactivated T_H cell-derived Ia-specific molecules and those obtained with antigen-specific T cell clones imply that during an antibody response to a given antigen, binding of the T cell antigen receptor and Ia molecule to the antigen, possibly in the form of a ternary complex (21), imparts antigenic specificity to the response.

Finally, our data suggest that a T_H/APC interaction such as that which may occur in the presence of antigen between either T_H^{IA}-1 or T_H^{IA}-2 and an appropriate APC, elicits a secondary interaction or signal transfer between an APC and a B cell. Thus, T_H/APC interaction might stimulate the synthesis and secretion of an APC- and/or T_H cell-derived soluble product(s) that binds to and activates a B cell in an *I-A*-nonrestricted and either antigen-specific or nonspecific manner (9). The characterization of B cell-activating molecules released subsequent to T_H-APC interaction is currently under investigation.

Summary

An allogeneic effect factor (AEF) derived from mixed lymphocyte reaction (MLR) cultures of alloactivated A.SW (*H-2*^{s}) responder T cells and irradiated T cell-depleted A/WySn (*H-2*^{a}) stimulator spleen cells was fractionated on the basis of molecular size and charge into two *I-A*-restricted helper components. The cellular origin of these components is believed to be an Lyt-1$^+$2$^-$-activated responder T helper (T_H) cell. One alloreactive component, T_H^{IA}-1, recognizes allo-*I-A* determinants on an A/WySn antigen-presenting cell (APC). The other self-reactive component, T_H^{IA}-2, recognizes self-*I-A* determinants on an A.SW APC. The interaction of each of these components with the appropriate APC subsequently activates an in vitro primary anti-SRBC PFC response of either stimulator haplotype- or responder haplotype-derived B cells. These

data demonstrate that the activity of T_H^{IA}-1 and T_H^{IA}-2 is dependent on the genotype of the APC and not the B cell, and that the target cell of action of these AEF T_H components is an APC.

T_H^{IA}-1 and T_H^{IA}-2 are 68,000 mol wt single polypeptide chains that have an isoelectric point (pI) of 5.8 and 5.5, respectively. Their charge difference is not attributable to altered amounts of sialylation or phosphorylation, but probably is due to other forms of altered glycosylation and/or to changes in their amino acid sequence. They share ~80% of their tryptic peptides and likely constitute homologous but nonidentical molecules. Papain cleaves T_H^{IA}-1 and T_H^{IA}-2 into a 40,000 mol wt fragment. T_H^{IA}-1 and T_H^{IA}-2 may represent structurally very related but nonidentical secreted forms of activated responder T_H cell-derived receptors for allo-*I-A* and self-*I-A* determinants, respectively.

We thank Drs. R. James, M. Feldmann, and M. Letarte for their kind gifts of monoclonal antibodies and antisera; Ms. K. Louste for her expert assistance with some experiments and the maintenance and care of our mouse colony; and Ms. F. Teodoro for her devoted assistance with the preparation of this manuscript.

Received for publication 13 August 1982 and in revised form 22 December 1982.

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