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*

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Previously, we isolated and characterized an I-A-restricted alloreactive T helper (Tn) 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 Tn 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 Tn 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 Tn 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 (TnF) and a KLH-specific T cell suppressor factor (TsF) (3), respectively, were kindly supplied by Dr. R. James and Dr. M. Feldmann, Department of

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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; Tn, T helper cell; TnF, T cell helper factor; Ts, T suppressor cell; TsF, T cell suppressor factor; TPCK, 1(tosylamide-2-phenyl)-ethyl-chloromethyl ketone.
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-2b) responder T cells and irradiated (3,000 rad) A/WySn (H-2a) stimulator spleen cells that were either depleted (i.e., treated with anti-Thy-1.2 plus complement [C3]) 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 spectrofluorometrically, 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 125I 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 125I-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^-8 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 10^9 T cell-depleted B + APC or 10^8 isolated APC and 5 × 10^5 isolated B cells.

Results

Fractionation of AEF TH Components. Fig. 1A shows that fractions of AEF eluting from ACA 54 in eight different mol wt ranges, designated pools I–VIII, displayed TH
Fig. 1. (A) Assay of AEF activity after gel filtration on ACA 54. About 25 ml of AEF was concentrated to 3 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 (10⁸). 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 TH 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 TH activity with either 10⁸ A/WySn (●) or A.SW (○) T cell-depleted spleen cells. The peaks of TH 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 TH 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 TH activity. Interestingly, an H-2-restricted TH 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 TH activity to be I-A^k restricted. Little H-2-restricted TH activity for A.SW lymphocytes (i.e., self-reactivity) was evident at this stage of AEF fractionation.

Since ACA 54 pool V of Fig. 1A contained strong TH alloreactivity and very much
weaker TH 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 TH 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 TH 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 TH 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 TH 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 TH Components.** An attempt was made to improve the resolution of the putative alloreactive and self-reactive TH components of AEF and to identify the target cell of action of these TH 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 TH 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 TH 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^-1 and T^-2, respectively. The data indicate that T^-1 and T^-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^-1 and T^-2 have shown that APC of strains that express I-A^k or I-A^d determinants are required to detect T^-1 activity, whereas APC of strains that express I-A^s or I-A^d determinants are required to detect T^-2 activity (data not shown). No responses were obtained for either AEF TH component in the presence of APC of strains that express I-A^s or I-A^d determinants. The patterns of reactivity of T^-1 and T^-2 are most likely attributable to structural homologies that exist between I-A-encoded Ia antigens of different haplotypes (8). Although both T^-1 and T^-2 display alloreactivity, only T^-2 exhibits self-reactivity for the responder haplotype APC (Fig. 2). We shall therefore continue to refer to T^-2 as a self-reactive component. T^-1 and T^-2 were active only if added during the first 24 h of culture. A TH/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 TH component of AEF (9).
T_{B}^{H}-1 and T_{B}^{H}-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_{B}^{H}-1 and T_{B}^{H}-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_{B}^{H}-1 and T_{B}^{H}-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_{B}^{H}-1 was estimated to be about 900-fold (Table I). A similar result was obtained for T_{B}^{H}-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_{B}^{H}-1 and T_{B}^{H}-2 after the second step of chromatofocusing were ~2 and 1 μg, respectively. Since the greater proportion of the T cells (≥90%) present in the cultures used to produce AEF were of donor origin (see Materials and Methods), we assume that both T_{B}^{H}-1 and T_{B}^{H}-2 are responder T cell derived. To detect T_{B}^{H}-1 and...
Table I

Purification of T1H-1

| Stage of fractionation | Protein | Total activity* | Specific activity‡ | Purification fold |
|------------------------|---------|-----------------|-------------------|------------------|
| 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/10^6 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.

T1H-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 T1H activity (unpublished data). This lends further support to the notion that T1H-1 and T1H-2 are responder T cell-derived. Insofar as 2.5 x 10^8 activated responder T cells are required to generate 25 ml of AEF, it follows that on the order of 1 μg of T1H-1 and T1H-2 are each secreted and/or shed from 10^8 alloactivated T cells. These yields are maximum estimates since, although T1H-1 and T1H-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 T1H-1 and T1H-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 T1H-1 and T1H-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 10^5 APC and 5 x 10^5 B cells. This picomolar concentration range of activity is very similar to that previously found for I-J-restricted AEF T1H components (5), IL-1 (11), and IL-2 (12).

Biochemical and Serological Analysis of T1H-1 and T1H-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 T1H-1 and self-reactive T1H-2 components. T1H-1 and T1H-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 T1H-1 and T1H-2 (see Fig. 2) were radiiodinated and analyzed by I-D 10% SDS-PAGE under reducing conditions before and after digestion with papain (11, 13). Before digestion, T1H-1 (Fig. 3A) and T1H-2 (Fig. 3B) 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
previously reported (1). T\(\text{H}^{\text{A}}\)-1 and T\(\text{H}^{\text{A}}\)-2 therefore do not appear to possess any subunit structure. Papain cleaved T\(\text{H}^{\text{A}}\)-1 (Fig. 3 C) and T\(\text{H}^{\text{A}}\)-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 \(^{125}\text{I}\)-labeled peptides as well as residual free \(^{125}\text{I}\) 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\(\text{H}\) 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\(\text{H}^{\text{A}}\)-1 and T\(\text{H}^{\text{A}}\)-2 with other proteases such as pepsin and trypsin.

Since T\(\text{H}^{\text{A}}\)-1 and T\(\text{H}^{\text{A}}\)-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. \(^{125}\text{I}\)-labeled T\(\text{H}^{\text{A}}\)-1 and T\(\text{H}^{\text{A}}\)-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\(\text{H}^{\text{A}}\)-1 and T\(\text{H}^{\text{A}}\)-2 (data not shown). The T\(\text{H}^{\text{A}}\)-1 cluster appeared in the pI 5.7–5.9 range and the T\(\text{H}^{\text{A}}\)-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\textsuperscript{H}{-1} and T\textsuperscript{H}{-2}, respectively. Treatment of T\textsuperscript{H}{-1} and T\textsuperscript{H}{-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\textsuperscript{H}{-1} and T\textsuperscript{H}{-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 radioacetylation (14) and/or from alternate forms of glycosylation cannot be ruled out.

To determine whether any of the charge heterogeneity between T\textsuperscript{H}{-1} and T\textsuperscript{H}{-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). \textsuperscript{125}I-labeled reduced and alkylated T\textsuperscript{H}{-1} and T\textsuperscript{H}{-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 \textit{I}-\textit{r}-restricted AEF T\textsubscript{H} components (5).

\textsuperscript{125}I-labeled tryptic peptides were separated on IEF gels using a pH gradient of 4–9.7 (Fig. 4). Nine peptides of T\textsuperscript{H}{-1} and eight peptides of T\textsuperscript{H}{-2} were observed. Seven of nine peptides (\textasciitilde80\%) are shared between T\textsuperscript{H}{-1} and T\textsuperscript{H}{-2}. T\textsuperscript{H}{-1} contains

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![Fig. 4. Tryptic peptide maps of \textsuperscript{125}I-labeled T\textsuperscript{H}{-1} and T\textsuperscript{H}{-2}. Aliquots (250 ng) of \textsuperscript{125}I-labeled T\textsuperscript{H}{-1} and T\textsuperscript{H}{-2} were reductively methylated, alkylated, and digested with 10 μg of \textit{L}(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone (TPCK) trypsin in 0.1 M NH\textsubscript{4}HCO\textsubscript{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\textsuperscript{H}{-1} and T\textsuperscript{H}{-2} peptides that differ from each other are designated by arrows.](image-url)
### Table II

**Serological Analysis of T\(^{a}\)-I and T\(^{a}\)-2**

| Antibody                  | CPM precipitated* |
|---------------------------|-------------------|
| AF.3.44.4 (anti-T\(\alpha\)F) | 492               |
| F 30.60 (anti-T\(\alpha\)F)    | 485               |
| Normal rat serum\(^{+}\)     | 347               |
| None\(^{+}\)                 | 228               |

* To 80 \(\mu\)l of PBS containing 50,000 cpm of either T\(^{a}\)-I or T\(^{a}\)-2 was added either 20 \(\mu\)l of anti-T\(\alpha\)F ascites (330 \(\mu\)g IgM/ml) or 1 \(\mu\)l of anti-T\(\alpha\)F ascites (16 mg IgG/ml). Reaction mixtures were incubated overnight at 4°C, 20 \(\mu\)l 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 \(\mu\)l 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 y-counter.

| Control samples | Result |
|-----------------|--------|
| 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\(^{a}\)-2, and T\(^{a}\)-2 contains one peptide absent from T\(^{a}\)-1. Thus, it seems that T\(^{a}\)-I and T\(^{a}\)-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-\(\alpha\)-restricted, AEF T\(\alpha\) component demonstrated that it does not bear any Ia, V\(\alpha\), C\(\alpha\) or L chain determinants. Similar results were obtained with the T\(^{a}\)-I and T\(^{a}\)-2 isolated and characterized in this study. Other studies conducted with heteroantisera and monoclonal antibodies, produced against either T\(\alpha\) or T\(\beta\) 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\(^{a}\)-I and T\(^{a}\)-2 for their expression of such putative isotypic determinants.

Aliquots of \(^{125}\)I-labeled T\(^{a}\)-I and T\(^{a}\)-2 were incubated with rat monoclonal antibodies reactive with either a KLH-specific T\(\alpha\)F or a KLH-specific T\(\beta\) factor (T\(\beta\)F) (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\(\alpha\)F and anti-T\(\beta\)F. T\(\beta\)F does not share epitopes with T\(\alpha\)F (3) and would not be expected to share epitopes with T\(^{a}\)-I and T\(^{a}\)-2. Although rat anti-T\(\alpha\)F does not react with T\(^{a}\)-I and T\(^{a}\)-2, the possibility remains that T\(\alpha\)F, T\(^{a}\)-I, and T\(^{a}\)-2 express another as yet undetected epitope(s) in common.

**Discussion**

The I-\(\alpha\)-restricted alloactivated responder T\(\alpha\) 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\(\alpha\) 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$\text{H}$ component (T$\text{H}^k$-1) of 68,000 mol wt and pI 5.8 from an I-A$^s$-restricted self-reactive component T$\text{H}^s$-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$\text{H}^k$-1 and T$\text{H}^s$-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$\text{H}^k$-1 and T$\text{H}^s$-2 to remove contaminating IL-2. Elution of T$\text{H}^k$-1 and T$\text{H}^s$-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$\text{H}^s$-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$\text{H}$ activity, perhaps by denaturing T$\text{H}^k$-1 and T$\text{H}^s$-2 and lowering their relative affinity for their respective I-A determinants. Since T$\text{H}^k$-1 and T$\text{H}^s$-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$\text{H}^k$-1 and allo-Ia determinants may be higher than that between T$\text{H}^s$-2 and self-Ia determinants. Although T$\text{H}^k$-1 and T$\text{H}^s$-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

| Property                      | T$\text{H}^k$-1  | T$\text{H}^s$-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$\text{H}$ cell | Lyt-1$^+2$-T$\text{H}$ cell |
| Target cell                   | I-A$^k$ APC     | I-A$^k$ APC     |
| Recognition of I-A determinants | Allo-I-A$^k$    | Self-I-A$^k$    |
| Expression of Ia determinants | No              | No              |
recovery with suitably high affinity for their target antigens.

Peptide-mapping analyses revealed that T~A~-1 and T~A~-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~A~-1 and T~A~-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~A~-1 and T~A~-2 are 90-95% homologous in their amino acid sequence. Since T~A~-1 and T~A~-2 were each purified probably greater than 900-fold before being analyzed by peptide mapping, it appears that T~A~-1 and T~A~-2 are homologous but nonidentical proteins. Further purification and characterization of T~A~-1 and T~A~-2 should confirm whether T~A~-1 and T~A~-2 are structurally different.

The T-A-restricted T~A~-1 and T~A~-2 polypeptides differ structurally from the previously described I-J-restricted self-reactive (T~J~-1) and alloreactive (T~J~-2) AEF TH components produced across an I-J incompatibility (5). Whereas T~J~-1 and T~J~-2 share 80% of their tryptic peptides, these proteins share only 50-60% of their tryptic peptides with either T~A~-1 or T~A~-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~A~-1 and T~A~-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~A~-1 and T~A~-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 Ts 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 Ts molecule 45,000 mol wt fragment specifically suppresses and lacks antigen-binding activity while the Ts 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~A~-1 and T~A~-2 40,000 mol wt fragments do not have any TH 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~A~-1 and T~A~-2 is most probably a 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 TH activity bear the Lyt-1^+2^- surface antigen phenotype (6). It shall prove interesting to determine
whether $T_{H}^{A-1}$ and $T_{H}^{A-2}$ 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 $T_{H}^{A-1}$ and $T_{H}^{A-2}$ demonstrated that $T_{H}^{A-1}$ recognizes allo-I-A-encoded determinants on an APC of the stimulator haplotype and that $T_{H}^{A-2}$ 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 $T_{H}^{A-1}$ and $T_{H}^{A-2}$ 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 $T_{H}^{A-1}$ or $T_{H}^{A-2}$ 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 $(H-2^a)$ responder T cells and irradiated T cell-depleted A/WySn $(H-2^b)$ 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, $T_{H}^{A-1}$, recognizes allo-$I-A$ determinants on an A/WySn antigen-presenting cell (APC). The other self-reactive component, $T_{H}^{A-2}$, recognizes self-$I-A$ determinants on an A.SW APC. The interaction of each of these components with the appropriate APC subsequently activated 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\textsuperscript{H}-1 and T\textsuperscript{H}-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\textsubscript{H} components is an APC.

T\textsuperscript{H}-1 and T\textsuperscript{H}-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\textsuperscript{H}-1 and T\textsuperscript{H}-2 into a 40,000 mol wt fragment. T\textsuperscript{H}-1 and T\textsuperscript{H}-2 may represent structurally very related but nonidentical secreted forms of activated responder T\textsubscript{H} cell-derived receptors for allo-I-A and self-I-A determinants, respectively.

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