H-2-RESTRICTED HELPER FACTOR SECRETED BY
CLONED HYBRIDOMA CELLS*

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Antigen-specific T helper and T suppressor factors are characterized by their
affinity to antigen and by determinants controlled by immunoglobulin heavy chain
variable region (V_H)[1] and *H-2I* loci (for reviews, see 1–3). A generally accepted
hypothesis suggests that specific T cell factors may be secreted receptors of T cells. It
is interesting, however, that whereas the function of most T cells is *H-2* restricted,
many antigen-specific T cell factors are not *H-2* restricted (4–14). It is not clear,
therefore, whether some *H-2*-restricted T cells produce nonrestricted factors, which
thus do not completely represent their receptor, or alternatively, the different factors
may respectively represent restricted and nonrestricted T cell sets. Because the
difference between regular and associative recognition most likely depends on the
nature of the receptor, this problem has important structural and genetic implications.
Analysis of the question involves studying *H-2* restriction of both the cellular receptor
and the helper factor from the same T cell clone. Hybridoma clones secreting *H-2*-
restricted (15) or nonrestricted (11–13) suppressor factors have been described. Much
less success was achieved, however, in the construction of helper factor-producing
hybridomas, and for neither case is there information that would compare factors and
receptors.

Recently we have described an approach for the preparation of helper hybridoma
cultures (16). One of a number of clones was characterized, and it was shown that this
clone, T85-109-45, secretes high titers of carrier, chicken gamma globulin (CGG),
specific helper factor. It has also been demonstrated that the T85-109-45 factor
contains Ia determinants and also determinants shared with the Ig V_H framework
(16).

This previous study concentrated on the antigen-binding properties of T-85-109-45

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[1] *Abbreviations used in this paper:* CGG, chicken gamma globulin; IAC, Ia-associated antigen complex
released by adherent cells incubated with antigen; NIP, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl; V_H,
immunoglobulin heavy chain variable region; V_λ, immunoglobulin λ-light chain variable region; V_κ,
immunoglobulin κ-light chain variable region.

cells, and it was reported that they bound CGG in an H-2-restricted manner after it was released in Ia-associated form by adherent cells preincubated with CGG (IAC; see also 17). Hence, this clone appears to carry H-2-restricted cellular antigen-binding receptors. This information is complemented here with more detailed data on the V_H and Ia determinants of factor T85-109-45, and it will be demonstrated that its helper effect is H-2-restricted.

Materials and Methods

Mice. For most studies (C3H/eB × C57BL/6J)F_1 ($H-2^{k/b}$) mice were used. For the genetic studies, congenic strains of independent haplotypes were used: C3H/DiSn ($H-2^k$), C3H.SW ($H-2^b$), C57BL/10J ($H-2^b$), B10.BR ($H-2^k$), B10.D2n ($H-2^d$), B10.Q ($H-2^q$) and B10.S ($H-2^s$), as well as recombinant strains: B10.A(2R) ($H-2^{h2}$), B10.A(4R) ($H-2^{h4}$), B10.HTT ($H-2^{t3}$), B10.S(9R) ($H-2^{t4}$), and A.TL ($H-2^{t1}$). Both sexes were used at the age of 2-3 mo. The mice were bred in the Laboratory Animal Breeding Center of the Weizmann Institute.

In Vitro Helper Assay. A carrier-hapten cooperation assay of CGG-primed nylon wool-purified (18) T cells and (4-hydroxy-5-iodo-3-nitrophenyl)acetyl- (NIP)-ovalbumin-primed and boosted spleen cells in the presence of NIP-CGG was used. Antibody production was detected by enumerating hemolytic plaques against NIP-sheep erythrocytes. The helper factor was measured by using it to replace syngeneic CGG-primed T cells. Details of the assay have been described previously (16, 17).

Construction of the Hybridoma Cells. Clone T85-109-45 was derived by limiting dilution cloning from line T85-109, which is a somatic cell hybrid between NIP-CGG immune B10.BR splenic T cells and BW-5147 AKR lymphoma cells; hence it is homozygous for $H-2^k$. The fusion and selection of our lines has been described previously (16). The hybridoma cells were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum. As helper factor, supernates from cultures close to saturation density (1.5-2.5 × 10^6 cells/ml) but not containing >10% trypan blue staining cells were used. Helper factor containing culture supernates or their affinity-purified fractions could be stored at −80°C for several months.

Serological Reagents. Alloantisera and monoclonal antibodies specific to different H-2 complex products are presented in Table I. The anti-H-2 sera were gifts from Dr. Edna Mozes (The Weizmann Institute). Monoclonal antibody 10.2.16 was a gift of Dr. L. A. Herzenberg (Stanford University). For more detailed description of the monoclonal antibodies, see references 19 and 20. Affinity-purified rabbit antibodies to V_H, immunoglobulin λ-light chain variable region ($V_λ$, and immunoglobulin κ-chain variable region ($V_κ$) have been described (21-23).

Table I
Anti-H-2 Antibodies Used in This Study

Designation	Immunization	Ig class	Subregion	Specificity	Correlation*	Form used‡
Anti-H-2^k	C3H.SW anti-C3H/DiSn	—	Whole H-2^k	—	—	Serum (NH$_4$)$_2$So$_4$ purified
Anti-H-2^b	C3H/DiSn anti-C3H.SW	—	Whole H-2^b	—	—	Serum (NH$_4$)$_2$SO$_4$ purified
Anti-Ia^k	A.TH anti-A.TL	—	Whole I^k, S^k, G^k	Ia.1, 2, 7, 17, 18, 19, 22, 31, 32, 33	—	Serum (NH$_4$)$_2$SO$_4$ purified
H118-49R1	BALB/c anti-C3H	μ, κ	I-A^k	Ia.m1	Ia, 2§	Culture-derived
B15-124R4	BALB/c anti-AKR	μ, κ	I-A^k	Ia.m2	Ia.2	Culture-derived
10.2.16	CWB anti-C3H	γ2b, κ	I-A^k	?	Ia.17	Culture-derived
13/18	A.Th anti-A.TL	γ2A, κ	I-E^k	Ia.m7	Ia.7	Ascites-derived
B17-123R2	AKR anti-C57BL/6	μ, κ	I-A^b	Ia.m4	No correlation	Ascites-derived
B17-263R1	AKR anti-C57BL/6	μ, κ	I-A^b	Ia.m3	No correlation	Ascites-derived
B22-277R19	BALB.K anti-C57BL/6	γ2A, κ	I-A^b	Ia.m8	Ia.8	Ascites-derived

* Correlation with determinants defined by conventional sera.
‡ For details see Materials and Methods.
§ Correlation incomplete.

Affinity Chromatography. The different proteins were coupled to Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Uppsala, Sweden) after cyanogen bromide activation. CGG was prepared from the serum of roosters by ammonium sulfate precipitation and DEAE (DE52; Whatman Inc., England) chromatography. Human gamma globulin was purchased from Sigma Chemical Co., St. Louis, Mo. Alloantisera were purified by ammonium sulfate precipitation. Monoclonal antibodies in ascites form were purified in the case of IgG antibodies by ammonium sulfate and in the case of IgM antibodies by boric acid precipitation. Culture-derived monoclonal antibodies were purified either on protein A-Sepharose (Pharmacia Fine Chemicals) or on anti-mouse Ig-Sepharose columns. The coupling ratio was 10–30 μg protein/ 1 g Sepharose. Hybridoma culture supernates were applied to the affinity columns at a dilution of 1:20, and they were eluted after extensive washing with 0.1 N NH$_4$OH into 0.5 M NaH$_2$PO$_4$ to achieve immediate neutralization. The eluates were dialyzed against RPMI 1640 medium for 2 h before use.

Adsorbtion of the Helper Factor to Spleen Cells. Culture supernates at a dilution of 10^{-2} or 10^{-3} in 0.5-ml vol were adsorbed on 10^8 spleen cells of normal mice at 4°C for 2 h.

Results

Composition of Helper Factor T85-109-45. To investigate the involvement of different variable regions, anti-V$_H$, anti-V$_\lambda$, and anti-V$_\kappa$-Sepharose columns were used. Results in Table II demonstrate that anti-V$_H$ retained the helper activity, which was regained in the eluate, whereas no significant activity was found in the column effluent. In contrast, anti-V$_\lambda$ and anti-V$_\kappa$ did not retain helper activity, and all of the activity appeared in the column effluent. These data are in agreement with a number of other results obtained with our anti-V$_H$ region reagents (24–26) or with anti-idiotypic antibodies (27–29).

Different Ia-antigenic determinants in the helper factor were investigated by affinity separation. Fig. 1A demonstrates that the helper activity was retained by anti-H-2^k and anti-Ia^k but not by anti-H-2^b or anti-Ia^s Sepharose, confirming previous suggestions that the factor contains determinants coded by the I^k region (16).

More detailed analysis was performed with monoclonal antibodies that react with Ia specificities of the k haplotype (Fig. 1B). Data obtained with three individual monoclonal antibodies have conclusively demonstrated that this helper factor contains determinants Ia.m1, Ia.m2, and Ia.17, which are controlled by the A^k subregion. It is of interest, however, that not all monoclonal I-A^k-specific antibodies retained this helper factor. Thus, the factor was Ia.m5 (17-227R7) and Ia.m6 (H116-32R5) (19) negative (data not shown). Furthermore, Fig. 1B demonstrates that the helper factor also binds to a monoclonal I-E-specific antibody that was purified from ascites fluid.

TABLE II

Helper Factor Contains Determinants Cross-reactive with Immunoglobulin V$_H$ but Not with V$_\lambda$ or V$_\kappa$

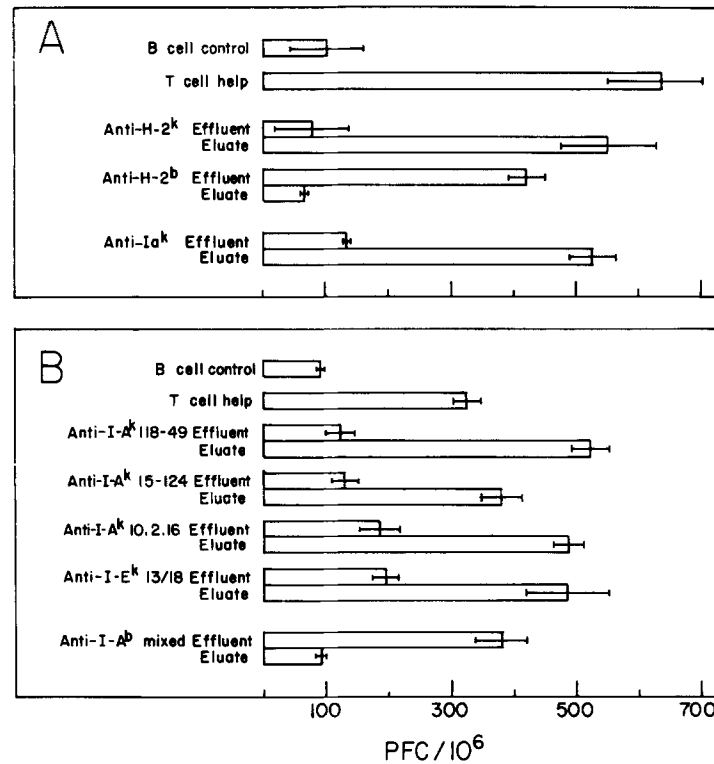|	PFC/10^6
B cell control	73 ± 11
T cell help	394 ± 37
Helper factor, anti-V$_H$-Sepharose effluent	80 ± 35
anti-V$_H$-Sepharose eluate	388 ± 35
anti-V$_\lambda$-Sepharose effluent	314 ± 33
anti-V$_\lambda$-Sepharose eluate	106 ± 6
anti-V$_\kappa$-Sepharose effluent	364 ± 47
anti-V$_\kappa$-Sepharose eluate	117 ± 24

FIG. 1. Ia-antigenic determinants in the helper factor. (A) conventional antibodies; (B) monoclonal antibodies.

This antibody, 13/18, is specific for Ia.7, a specificity of the E subregion of $H\text{-}2^k$. To investigate the possibility that this ascites-derived antibody could have bound the factor nonspecifically, supernate of the hybridoma cultures was passed through an affinity column containing mixed immunoadsorbents of three $I\text{-}A^b$-specific ascites-derived monoclonal antibodies (Table I). The results supported the conclusion of the previous experiments by demonstrating that the helper activity was not retained on this affinity column (last two histograms of Fig. 1B).

Taken together, the data demonstrate that the helper factor of clone T85-109-45 expresses V_H-, $I\text{-}A$-, and most likely also $I\text{-}E$-controlled determinants.

H-2 Restriction of the Factor-Mediated Helper Activity. In a previous publication, we have reported the establishment of 11 T cell fusion lines (16). At that time, six of these had been tested and found to produce CGG-specific helper factors (16). Most of these lines were constructed from $H\text{-}2$-dissimilar parental cells. In contrast, line T85-109 was constructed from two $H\text{-}2^k$ type cells. Hence its clone, T85-109-45, which expresses both $H\text{-}2K$ and $H\text{-}2D$ gene products of the k haplotype (16), seemed to be an ideal tool to study $H\text{-}2$ restriction of the factor-mediated helper effect.

This question was analyzed by testing helper activity in culture supernates by the in vitro helper assay, using cells from different $H\text{-}2$-congenic mouse strains. Fig. 2 demonstrates the results. The three histograms at the top of the figure demonstrate factor-mediated helper activity with (C3H × B/6)F$_1$ ($H\text{-}2^{k/b}$) cells, which were used in the previous experiments to test the helper factors of our various hybridomas. The
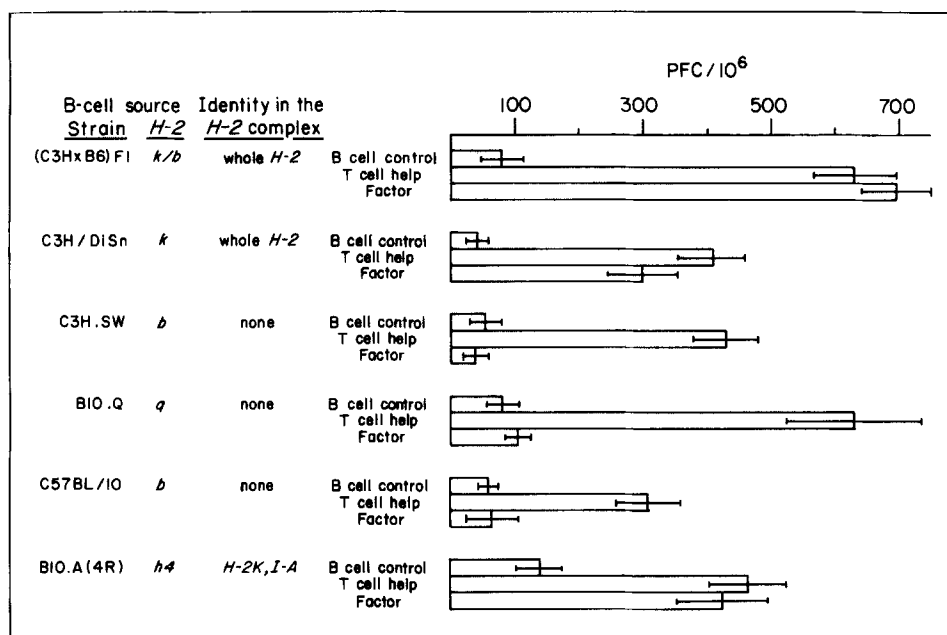
Fig. 2.   H-2 restriction of the factor-mediated helper effect.

following two groups of histograms show results with two congenic strains, C3H/DiSn (H-2^k) and C3H.SW (H-2^b). It can be seen that, whereas the factor was active with cells of the strain carrying the H-2^k haplotype, homologous with T85-109-45 cells, no activity was obtained with cells of the b haplotype, which are allogeneic to the hybridoma cells. Three more haplotypes on C57BL/10 background were also investigated. The data demonstrate that with the allogeneic, q, and b haplotypes, no helper activity was obtained. The last group of histograms shows that the helper factor induced significant antibody production in cultures derived from B10.A(4R) inter-H-2-recombinant mice. This haplotype h^4 is a recombinant between haplotypes b and a and has H-2^k loci in the K and I-A regions, whereas the rest of its H-2 complex was derived from the b haplotype. Hence, the data suggest that for helper effect by this monoclonal factor, H-2 identity between the factor and the B cell source is required, and that it is sufficient if the identity is restricted to the K and I-A regions of H-2.

Cellular Absorption of the Helper Factor.   Helper factors have an antigen-specific effect that results in the stimulation of antibody production by B cells. It is obvious that their target cells should have receptors or acceptor sites for the binding of helper factors. We have investigated the genetic regulation of adsorption of the helper factor to spleen cells.

Diluted hybridoma culture supernates were adsorbed on spleen cells of nonimmunized mice from different congenic strains. Preliminary experiments have suggested (not shown) that adsorption of the helper factor requires H-2 identity between the spleen cells and the factor producing hybridoma cells. This observation was used as a basis for a mapping study by which we aimed to establish the map position of loci responsible for helper factor binding.

Fig. 3 demonstrates the results of helper factor adsorption by spleen cells from a

series of congenic and recombinant strains. The data demonstrate that *H-2* identity is necessary for absorbtion. Results with strains B10.A(4R) and B10.HTT as well as with B10.S(9R) suggested that the absorbtion of the factor is associated with the left part of the *H-2* complex similarly to the map position of the *H-2* restriction of the helper effect. Finally, data with A.TL absorbing cells, when compared with those obtained with B10.A(4R) cells, have shown that the absorption of the helper factor is regulated by loci in the *I-A* subregion.

If we compare the results presented in Figs. 2 and 3, it appears that the two phenomena connected to factor-mediated helper effect, *H-2* restriction of helper function and the restriction of cellular adsorption, are controlled by closely linked loci in the left part of the *H-2* complex.

Discussion

The present study describes an *H-2*-restricted, carrier-specific helper factor from cloned hybridoma cells. The data leading to this observation demonstrated that to obtain helper activity with this factor, the genotype of the hapten-primed B cell source has to carry loci in the left part of *H-2* that are identical to those of the *H-2* homologous parental cells of the hybridoma. Previous experiments with the same clone demonstrated that specific binding of IAC (17) derived from macrophages incubated with the carrier, CGG, is also *H-2* restricted (16). Hence, it appears that the helper factor and the cellular antigen receptor of T85-109-45 are both characterized by similar dual specificity for CGG and self-*H-2I* gene products.

Results of these two studies also suggest that both the helper factor and the cellular antigen receptor contain determinants that are recognized by V_H framework-specific antibodies (16 and present study). Moreover, the two studies demonstrate that neither the receptor nor the helper factor reacts with antibodies specific to the V region of κ or λ light chains. The reagents used in these experiments were extensively characterized by serological (21–23) as well as functional experiments. It has been shown that anti-V_H but not anti-V_L inhibits antigen binding by Lyt-$1^+,2^-,3^-$ normal lymphocytes (24); that anti-V_H but not anti-V_λ inhibits streptococcal carbohydrate-specific helper cells (25), and also that anti-V_H but not anti-V_λ or anti-V_κ protects helper T cells from radioactive antigen "suicide" (26). Additional studies have shown that anti-V_H binds the Ig^- receptor of hapten-specific T cells (30), that it interferes with the binding of membrane receptors by allostimulated T cells (31), and that it is present on the
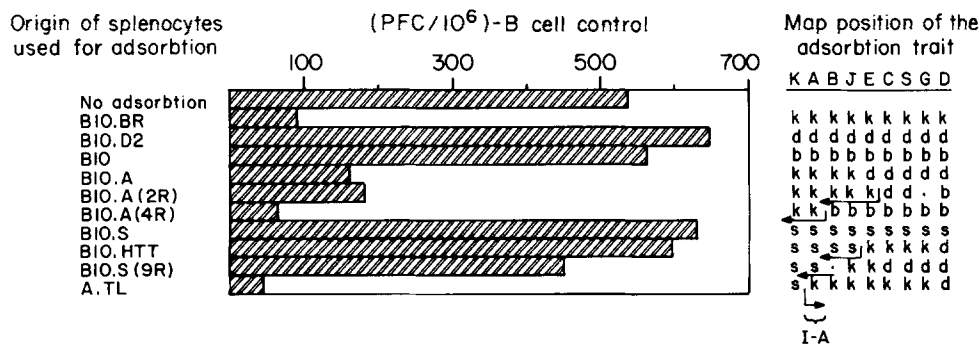

FIG. 3. Cellular adsorption of the helper factor.

membrane of certain suppressor (32) and helper-factor-producing hybridomas (33). It has also been demonstrated that anti-V_H, which is a rabbit antibody to the V_H fragment of myeloma protein M-315 of BALB/c mice, reacts with allelic determinants probably not shared with heavy chains of AKR mice. This allelic determinant was shown to segregate with *Igh-1* allotype (34). This suggests that anti-V_H indeed reacts with immunoglobulin V gene products. Hence, our data complement those that demonstrate shared heavy chain-specific idiotypic determinants in antigen-specific T cell receptors and factors (25, 26, 29); moreover, because anti-V_H reacts with nonidiotypic determinants, they suggest that possibly a major portion of the Ig V_H region may be present in the helper factor.

The previous and present data taken together strongly emphasize similarities between the soluble factor and the cellular antigen receptor of helper T cells. Since the discovery of antigen-specific suppressor and helper factors, it was widely believed but was not directly demonstrated that specific T cell factors represent secreted receptors (1). Our data provide evidence for this hypothesis.

The helper factor of T85-109-45 cells contains Ia-antigenic determinants. Our results revealed that this helper factor carries Ia.m1, Ia.m2, Ia.17, and Ia.m7 specificities. The first three of these specificities are controlled by the *A* subregion (19, 20), and they were detected with antibodies isolated from culture supernates of B hybridoma cells. Hence, they most likely did not contain irrelevant, e.g., retrovirus-specific, antibodies. In contrast, Ia.m7, which is controlled by the *E* subregion (19), was detected by ascites-derived antibodies. The specificity of these results is, however, supported by the fact that three monoclonal *I-A^b*-specific ascites-derived antibodies did not bind this *H-2^k* type helper factor.

These data are consistent with the minimal hypothesis that this helper factor contains Ia-antigenic determinants controlled by both *Aβ* and *Eα* loci (35).

For a more comprehensive analysis, additional monoclonal anti-Ia antibodies will have to be studied. The Ia chain specificity of these antibodies will also have to be known. Then it will be possible to determine which chain combinations are involved, and the theoretical possibility that the cell produces both Aβ + Eα- and Aβ + Aα-containing factors could be judged. In this respect it may be important that this factor is Ia.m5 and m6 negative, which may suggest that helper T cell clones utilize specific combinations from the available Ia phenotype. Finally, biochemical studies will be necessary to determine whether the Ia positivity of these factors signifies only Ia determinants, possibly carbohydrates (36), or complete Ia polypeptide chains.

The role of Ia antigens in antigen-specific T cell factors is not clear. Moreover, in the case of *H-2*-restricted factors, one cannot rule out the fact that the Ia portion could be bound by an anti-self receptor, and thus may have no functional significance (37). Nevertheless, a number of data suggest that they may be involved in factor-target cell interaction (for reviews, see 1–3). Our cellular absorbtion experiments support this view. They demonstrate (Fig. 3) that the absorbtion of the factor to normal spleen cells is controlled by loci in the *I-A* subregions, similarly to the *H-2* restriction of the helper effect. Both findings may be causally connected with the presence of *I-A*-controlled determinants on the helper factor. Absence of *I-E*-linked restriction in the helper effect and in cellular absorbtion, despite the possible presence of *I-E* determinants on the helper factor, may be explained by the lower degree of structural polymorphism of *I-E* products as compared to *I-A* products (35, 38). The

exact role of helper-factor Ia determinants in their *H-2*-restricted activity remains to be determined. It is possible that *H-2*-restricted helper factors recognize Ia on the absorbing cells by an anti-self receptor. Alternatively, Ia determinants of the factors could be recognized by Ia-controlled interaction determinants (39).

Two kinds of antigen-specific suppressor and helper factors were detected in different experimental systems (1–3). Accordingly, both *H-2*-restricted and nonrestricted helper and suppressor factors may exist. All of these factors function in the presence of the specific antigen and bind it in Sepharose-coupled form. This is compatible with regular antigen recognition. Nevertheless, *H-2*-restricted receptors could also recognize Sepharose-bound antigens coupled at high density via low affinity, anti-"nominal" antigen receptors. Hence, the present data suggest that the specificty of *H-2*-restricted T cells and their factors is similar. Antigen recognition by nonrestricted factors and T cells (40, 41) may also be based on similarities between the respective factors and receptors. Because stringent connection between T cell receptors and factors can be made only when the factor and receptor of the same cell are compared, the problem of nonrestricted factor-producing T cells can be solved only by appropriate cellular antigen-binding assays. The present study and a previous study (16) taken together demonstrate this approach for *H-2*-restricted helper cells and factors. Biochemical and immunochemical studies of such *H-2*-restricted helper factors will further our understanding of associative recognition.

Summary

Biological and serological characteristics of a helper factor secreted by cloned hybridoma cells was described. The factor is carrier specific and contains determinants shared with immunoglobulin V_H but does not react with V_κ- or V_λ-specific antibodies. Presence of four *H-2I*-controlled antigenic specificities, Ia.ml, Ia.m2, Ia.17, and Ia.m7, was detected. Hence, it is possible that both *Aβ* and *Eα* loci may be involved in its control. Helper effect could be obtained only toward B cell sources that shared the *H-2K* and *I-A* regions with the hybridoma cells. Similarly, the factor was absorbed only by spleen cells syngeneic in *I-A*. Previous studies have demonstrated that this clone binds antigen in an *H-2*-restricted manner. It follows that *H-2*-restricted helper cells produce *H-2*-restricted helper factors. Hence, they support the view that specific T cell factors may represent secreted T cell receptors.

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