ANTIGEN-SPECIFIC SOLUBLE HELPER ACTIVITY
FOR MURINE MAJOR HISTOCOMPATIBILITY
COMPLEX-ENCODED MOLECULES

I. Kinetics of Factor Production after
Skin Transplantation and Genetic Mapping
of the H-2 Region Specificity*

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A basic, fundamental feature of an antigen-stimulated antibody response is the
specificity of the binding capacity of the antibodies produced for determinants on
that stimulating antigen. The exquisite specificity that is evidenced by antibodies for
their antigenic determinants is also observed in T cell-mediated killing responses
directed against cell surface antigenic molecules. The major target molecules for such
cytotoxic T effector cells are associated with the major histocompatibility complex
(MHC), which is the H-2 complex in the mouse. Although "cross-reactive" killing of
cellular targets obtained from mice with different H-2 haplotypes is frequently
observed, recent evidence suggests that such cross-reactivity arises through the acti-
vation of distinct prekiller cell clones. Although the majority of the cell clones reactive
to MHC antigens recognize the immunodominant specificity that is distinct for a
given haplotype ("private" specificity), other distinct prekiller cell clones are specific
for MHC determinants that are shared by a number of different H-2 haplotypes
("public" specificities) (1-4). Thus, the development of cytolytic effector T cells
directed against cell surface H-2 antigenic determinants appears to follow the laws of
clonal selection and immunological specificity. Similar rules concerning the generation
of antigen-specific help for T cytolytic effector cell development have not been readily
evident. T cell help for alloreactivity can be generated with virtually any H-2I-region

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1 Abbreviations used in this paper: CFA, complete Freund's adjuvant; Con-A, concanavalin A; Co, control
cultures; HFS, nonspecific helper factor supernatants generated in vitro; IL-2, interleukin 2; LNC, lymph
node cells; MHC, major histocompatibility complex; MLR, mixed lymphocyte culture reactions;
18R, B10.A(18R); 7R, B10.S(7R); oz1, B6.H-2oz1; DM1, B10.D2.H-2m1; DM2, BALB/c-H-2m2; B10,
C57Bl/10; 2R, B10.A(2R); SgHF, skin graft-induced helper factor.
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difference (5). Such help is not specific for the prekiller T cell clones that respond to either the H-2K and/or H-2D region cytotoxic T cell targeted specificities. Thus, nonspecific help provides an adequate signal for specific prekiller cell differentiation.

Alloantigen-specific helper T cells have been suggested, however, as a requirement for the generation of both antibody responses and of cytotoxic T cells derived from thymocyte precursors (6, 7). Alloantigen-specific helper T cells in both of these studies were obtained from mixed lymphocyte culture reactions (MLR). Long-term primed MLR cells were also demonstrated (8) to be capable of releasing materials that upon transfer into athymic nude mice could specifically help antibody responses to antigens of the MLR-stimulating H-2 haplotype. These latter findings have not yet been confirmed or extended. In general, soluble help derived from short-term MLR culture supernatants has lead to the demonstration of nonspecific helper effects for both T and B effector cell development (9-13). Cloned antigen-specific helper T cell lines derived from MLR cultures or, for that matter, any antigenic stimulus, all produce nonspecific soluble help in vitro. We also have not been successful in generating MHC antigen-specific soluble help in vitro even when we have used either of the above short- or long-term culture procedures where MHC antigen-specific helper cells have been defined. Such overwhelming evidence for the production of nonspecific soluble helper factors has led to a generalized belief that antigen-specific helper cells, upon contact with that antigen, do not release antigen-specific helper molecules. Rather, they release, amongst a variety of factors, helper molecules that are not specific for that antigen but also help responses to other antigens in a bystander type effect (14-16).

Most studies demonstrating nonspecific helper factor production have been performed in vitro; thus, we sought to investigate the possibility of generating MHC antigen-specific soluble help in vivo during the development of immune responses to skin allografts. The kinetics of factor production by cells in the lymph nodes draining the sites of the skin allotransplants were evaluated, and the dynamics of the response proved to be most interesting. MHC antigen-specific soluble help for the generation of cytotoxic effector T cells was evident as a relatively early event in the development of the immune response. The specificity of the soluble helper activity maps to the classical MHC regions, whose molecular products serve as targets of effector T mechanisms both in vivo and in vitro. In the strain combination chosen for our study, both helper and effector targets mapped to the H-2D region. The early phase of antigen-specific soluble help was followed by an intermediate phase, during which we found lower levels of helper activity and preliminary evidence for the production of suppressor activity. The antigen specificity and/or targets of suppressive activity have not yet been identified. This intermediate phase was followed by a second phase of helper factor production that proceeded through graft rejection before waning. The helper activity detected during this second later phase contained largely cross-reactive help, nonspecific help, and interleukin 2 (IL-2) activity. We describe the dynamics of factor production by cells within lymph nodes draining the sites of skin transplants and examine in detail the antigenic specificities for which the early phase skin graft-induced helper factor supernatants (SgHF) can provide help.

Materials and Methods

Animals. The B10.A(18R) mice (18R) were raised locally (Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL) by strict brother-sister matings from breeding pairs provided by
Dr. Jack Stimpfing, McLaughlin Research Institute, Great Falls, MT. Dr. Stimpfing also provided the B10.S(7R) mice (7R). The B6.TL* and B6.H-2pos mice (oz1) were raised by Dr. Lorraine Flaherty, New York Department of Health, Albany, NY. The B10.M, B10.D2.H-2dm1(DM1), (B6 × DM2)F1, and BALB/c-H-2dm2(DM2) lines were raised by Dr. Roger Melvold, Northwestern University Medical Center, Chicago, IL. The DBA/2J, HTG/AoJ, B10.A(2R)SgSn, B10.D2nSn, B10.BRSgSn, and C57BL/10SnJ (B10) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. The H-2 genotypes of the recombinant and mutant strains are given in Table I.

**Generation of Helper Factor Supernatants.** Supernatants containing nonspecific helper factor activity (HFS) were obtained from two-step mixed lymphocyte culture procedures, as described previously (10). C57BL/10 responder strain lymph node cells (LNC) and B10.D2 gamma-irradiated spleen cells or vice versa were generally used for nonspecific HFS production. The supernatants containing SgHF were derived from 6-h cultures containing cells from the recipient strains’ draining lymph nodes plus an equal number of LNC from the skin donor strain. C57BL/10 mice and the congenic recombinant line B10.A(18R) served as recipients and donors, respectively, in the allotransplantation studies (Table I).

**Skin Grafting.** Full thickness body or tail skin grafts were performed according to the method of Billingham and Medawar (17). C57BL/10SnJ mice served as the recipients of B10.A(18R) skin. At 4, 5, 8, 10, 12, 14, 16, and 19 d after graft emplacement, the lymph nodes draining the graft sites were removed, teased apart with tuberculin syringe needles, and resuspended in RPMI 1640 medium at a concentration of 4 × 10⁶ cells/ml. Cell suspensions of B10.A(18R) lymph nodes were similarly prepared and an equal number was mixed with the recipient cells. The final cell suspensions (8 × 10⁶ cells/ml) were incubated for 6 h at 37°C in a humidified atmosphere of 5% CO₂. The supernatants were thereafter removed, filtered through a 0.45 μm Millipore filter (Millipore Filter Corp., Bedford, MA), aliquoted, and stored at -70°C.

**Assessment of Helper Factor Activity.** C57BL/10 recipient strain LNC at 10⁶ cells/microwell served as responders. Nucleated splenic cells from the skin graft donor strain B 10.A(18R) served as responders. Nucleated splenic cells from the skin graft donor strain B10.A(18R) served

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### Table I

**Major Histocompatibility Haplotypes of Target Specificities**

| H-2 phenotypes* | Qa-2 | Specificities in common with B10.A(18R) and foreign to C57BL/10 |
|-----------------|------|---------------------------------------------------------------|
| K | I | S | D,L | Qa-2 | Qa-1/ Tla |
| C57BL/10 | b | b | b | b,b | a | b/- (b) |
| B10.A(18R) | b | b | b | d,d | a | +/a |
| P815-X2 | d | d | d | d,d | - | ?/- (b) |
| B10.M | f | f | f | f,f | b | ?/+ (d) |
| B6.Tla* | b | b | b | b,b | a | +/a |
| B10.A(2R) | k | k/d | d | b,b | a | b/- (b) |
| B10.BR | k | k | k | k,k | b | a/+ (a) |
| B6.H-2pos | b | b | b | k,k | b | b/- (b) |
| B10.S(7R) | s | s | s | d,d | a | +/a |
| B10.D2-H-2dm1 | d | d | d | dm1,dm1 | ? | ?/- (b) |

* Taken from Klein et al. (20).
† Qa-1 may be detectable on P815-X2 tumor cells (R. Rich et al., personal communication).
§ Although peripheral lymphoid cells normally do not express TLa determinants, they might have become reexpressed on blast cells.
|| The DM1 mutation apparently is quite complex and involved a segment of chromosome that affects expression of both the D and L molecules (28).
as the source of antigenic stimulus. Cytotoxic effector T cells were generated during a 4- or 5-
day incubation period in culture with gamma-irradiated (2,000 rad from a 137Cs source) cells.
To assay for exogenous soluble helper activity, the normal generation of such killer cells was
prevented by glutaraldehyde fixation of the stimulating strain cells. The concentration of
 glutaraldehyde required to effectively block the normal generation of killer cells and yet allow
their development in the presence of exogenous soluble help varied with the responder-
stimulator strain combination. In the studies reported here, we used 10 ml of a 0.01, 0.02, or
0.05% glutaraldehyde solution (Eastman Kodak, Rochester, NY) and fixed the nucleated
splenic cells at 37°C for 3 min. The cell suspensions were then immediately diluted to 50 ml
with RPMI 1640 medium containing 10% human serum and washed three times before
culturing at either 0.5 × 10⁶ or 0.1 × 10⁶ viable cells/microwell. The cells were cultured in a
final volume of 0.2 ml of RPMI 1640 medium containing 10% human serum (heated at 56°C
for 30 min), 50 mM Hepes buffer plus 50 μg L-arginine, 75 μg L-glutamine, 100 U penicillin G,
and 75 μg kanamycin sulfate per ml. 2-mercaptoethanol, 10⁻⁴ M, was an absolute requirement
in cultures with glutaraldehyde-fixed cells. SgHF MLR-induced nonspecific HFS were added
at a final concentration of 20%. 5–10 replicate wells for each assessment were set up, such that
sufficient cells could be obtained after 4 or 5 d in culture to titrate the yield of killer activity
generated.

Assessment of the Generation of Killer Cells. The quantitation of killer cell activity obtained was
evaluated directly for each helper factor sample assayed in culture. An identical number of
cells was initially cultured in replicate wells with or without the exogenous source of helper
activity. The pool of viable cells recovered from the replicate wells was resuspended in 0.45 ml.
The killer cell activities obtained were determined directly by titrating each pool in duplicate
beginning with 0.1 ml of cell suspension. Numbers of recovered viable cells were counted with
the remaining 0.05 ml. This direct method of assessment allows us to present actual percentages
of specific ⁵¹Cr release rather than calculated cytolytic units generated per sample assayed for
a comparison of cells incubated, for example, without HFS, with HFS, and with SgHF.

DBA/2-derived, H-2a-bearing, P815-X2 mastocytoma and/or nucleated concanavalin A
(Con-A; Sigma Chemical Co., St. Louis, MO)-induced blast cells were labeled with 100 μCi of
⁵¹Cr (New England Nuclear, lmCi/ml) and washed in Dulbecco’s phosphate-buffered saline
(Grand Island Biological Co., Grand Island, NY), containing 3% calf serum. The ⁵¹Cr-labeled
target cells at 2.0 × 10⁶ cells/well were added to titrations of the killer cells, incubated at 37°C
for 4 h before 150 μl of supernatant was removed and ⁵¹Cr released thereto counted in a gamma
scintillation counter.

Killer cell activity within draining lymph nodes and spleens of skin graft recipients was
determined after a 16-h incubation period with ⁵¹Cr-labeled P815-X2 target cells.

The percent specific ⁵¹Cr release and percent enhanced ⁵¹Cr release were calculated as follows:

\[
\text{percent specific } ⁵¹\text{Cr release} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm total}} \times 100 \%
\]

\[
\text{percent enhanced } ⁵¹\text{Cr release} = \frac{\text{cpm experimental} - \text{cpm glutaraldehyde}}{\text{cpm total}} \times 100 \%
\]

IL-2 Proliferation Assay. The presence of IL-2 in our various helper factor supernatants was
evaluated by their ability to support the proliferation of antigen-primed LNC (18, 19). Ovalbumin
(OVA) was emulsified in complete Freund’s adjuvant (CFA), and 100 μl containing
100 μg OVA was injected subcutaneously at the base of the tail. Regional lymph nodes were
removed 7 d later, and cell suspensions of 6 × 10⁶ cells/2-ml macrowell were restimulated in
culture with 100 μg OVA for 4 d. The cells were then collected, washed, and resuspended at 1
× 10⁶ LNC/ml of complete medium. An equal volume of cell suspension, 0.1 ml/well, was
mixed with supernatant and further cultured for 4 d. The cells were labeled with 1 μCi
[³H]thymidine (Schwarz/Mann, Orangeburg, NY; sp act of 6 mCi/ml) for 18 h before
harvesting and counting. The data presented are an average of quadruplicate samples. The
FS6-14.13 T cell hybridoma was a generous gift from Dr. P. Marrack et al., Denver, CO.
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Results

Kinetics of Cytolytic Effector T Cell Development after Skin Allograft Transplantation. The generation of cytolytic effector cells in vivo was examined in HTG mice who had received a skin graft from B10.D2 strain donors. These recipients recognize B10.D2 strain cell surface antigens of the H-2D region as well as non-MHC-associated antigens (20). Such skin allografts were rejected by 11 d post-transplantation (unpublished observations). The detection of cytolytic effector cells followed the kinetic of skin graft rejection (Fig. 1). Peak cytolytic activity was attained after the time of graft rejection with cells obtained from either the draining lymph nodes or spleens. The development of cytolytic effector cells was detected earlier and for a broader period of time in draining lymph nodes (Fig. 1, left) than in spleens (Fig. 1, right). Significant levels of killer cell activity were not evident in a 4-h $^{51}$Cr release assay but increased after 6 h to maximum or total killing at 24 h (Fig. 2). The killer cell activity observed at both 13 and 15 d was mediated by T cells, as all activity was eliminated after treatment with an anti-Thy-1.2 serum [(AKR X RF)F1 anti-C58] plus complement.

Kinetics of Soluble Helper Factor Production after Skin Allograft Transplantation. Because the development of cytolytic effector T cells to the H-2D$^d$ region-determined antigens was demonstrable during and after allograft rejection, we sought to determine whether soluble helper factor was also produced to these same cell surface antigens during priming in vivo. We were particularly interested in the possible production of antigen-specific helper molecules; thus, we chose a congenic resistant recipient-donor strain combination in which the strains differed only for classical class I transplantation antigens and were identical for class II, I region-encoded antigens. The H-2D region lends itself more readily for such an analysis than does the H-2K region because a greater number of I-D region recombinant lines are available for specificity studies than are K-I region recombinants. The B10.A(18R) recombinant and one of its parental lines, C57BL/10, were selected as a combination that would meet these

![Fig. 1](image)

Fig. 1. The detection of cytolytic effector T cells during skin allograft priming in vivo. Left, draining LNC (○); and right, nucleated splenic cells (▲) from HTG recipients of B10.D2 skin grafts were assayed at optimum ratios of LNC (200:1) and splenocytes (50:1) against $^{51}$Cr-labeled H-2$^d$-bearing P815-X2 target cells for 15.5–17 h. The data are expressed as average and ranges of the activities obtained from two recipients assayed at each time point.
Fig. 2. Kinetics of $^{51}$Cr release mediated by allograft-primed cytotoxic effector cells. Cells from 6 HTG recipients: left, draining lymph nodes at 200:1 ($\mathbf{\square}$), 100:1 ($\mathbf{\bigtriangleup}$), and 50:1 ($\mathbf{\blacktriangle}$); and right, spleens at 100:1 ($\mathbf{\bullet}$), 50:1 ($\mathbf{\bigcirc}$), and 25:1 ($\mathbf{\blacklozenge}$) were assayed against P815-X2 target cells ($\mathbf{\bigtriangledown}$) 13 d after receiving B10.D2 skin allografts. $^{51}$Cr release in mixtures of P815-X2 cells with LNC and nucleated splenocytes from nongrafted HTG recipients coincided with spontaneous release at 6 h and 9 h and an average increase of 3.8 and 4.5% at 18 h and 24 h, respectively.

Fig. 3. Helper activity of SgHF for B10.A(18R) priming of C57BL/10 cytotoxic effector cell generation. The soluble helper activities were obtained from 6-h cultures of C57BL/10 draining LNC at the various days (D) indicated after allotransplants of B10.A(18R) skin or MLR in vitro (Co). The supernatants were assayed at a final concentration of 20% (black bars) and 10% (striped bars). Nonspecific HFS (Co) and gamma-irradiated B10.A(18R) cells served as positive controls. $^{51}$Cr-labeled P815-X2 tumor cells served as targets in these assays.

criterion. The B10.A(18R) line originated as a recombination between the H-2$^b$ and H-2$^a$ genotypes and differs from the C57BL/10 strain in the H-2D-TLa regions (Table I). B10.A(18R) strain skin donated to B10 recipients was rejected at ~12.5 d.

6-h supernatants were prepared from C57BL/10 recipient draining lymph nodes at various times after skin grafting. These supernatants were assayed for helper activity using glutaraldehyde-fixed cells as helper-dependent stimulators of cytolytic effector cell development. Skin allograft-induced production of helper factor activity was clearly evident as early as 4 d after transplantation (Fig. 3), when the B10.A(18R) skin donor strain fixed cells were used as stimulators. The higher concentrations of SgHF, in fact, suggested a biphasic kinetic response in helper factor production, with a final waning period ~1 wk after graft rejection. The biphasic kinetic response in
helper production was supported in antigen specificity studies with strains that express few or no antigenic determinants in common with those of the H-2D<sup>d</sup>,L<sup>d</sup> molecules.

**Antigen Specificity of SgHF Produced at Various Times during Allograft Priming.** Aliquots of the C57BL/10 anti-B10.A(18R) (B10 anti-18R) SgHF obtained at the various time points and tested with 18R-fixed cells were then assayed for specificity of soluble helper activity with different donor strains. The B10.M strain (H-2<sup>i</sup>) was chosen as a primary specificity control because this strain bears no known cross-reactive or public specificities in common with the 18R strain D<sup>d</sup> region (Table I) (21). Little evidence of helper activity for the B10.M strain was demonstrated in the SgHF induced during the early phase of allograft priming (Fig. 4). Helper activity was consistently detected, however, in supernatants obtained after graft rejection. The level of helper activity in 19d SgHF for the response to B10.M strain fixed stimulators approximated that obtained in control cultures (Co) incubated with nonspecific HFS generated in vitro (Fig. 4).

The B10.A(2R) recombinant strain was used as a second specificity control. It bears cross-reactive public antigens of the H-2K<sup>k</sup> in common with those of the H-2D<sup>d</sup>-defined molecules (21). The most significant levels of soluble helper activity detected for the B10.A(2R) strain fixed stimulator cells were observed during the second phase of helper factor production around the time of graft rejection (Fig. 5). Little helper activity for B10.A(2R) was observed with SgHF obtained during the early inductive phase of B10 anti-18R allograft priming. When cross-reactive public antigens of both the K<sup>k</sup> and D<sup>k</sup> molecules were presented by B10.BR strain fixed stimulator cells, however, a somewhat higher degree of helper activity was observed in the early induced first-phase SgHF preparations (Fig. 6). Again, an intermediate phase of lower helper factor activity was observed.

These data taken together suggest that upon initial 18R allograft priming, B10 draining LNC release helper molecules that are specific for determinants expressed by the H-2D-TLa region-encoded molecules. The lack of support provided by early phase SgHF helper activities observed for B10.M and B10.A(2R), as contrasted with 18R stimulator cells, strongly support this conclusion. Furthermore, the responses observed with early phase SgHF and B10.BR strain stimulating cells suggest that

![Fig. 4. Helper activity of B10 anti-18R-induced SgHF for B10.M priming of C57BL/10 cytotoxic effector cell generation. The various samples assayed were identical to those used in Fig. 3 (see legend to Fig. 3). Nonspecific HFS (Co) and gamma-irradiated B10.M cells served as positive controls. 51Cr-labeled B10.M blast cells served as targets for these assays.](image)
besides the specificity for the private H-2D<sup>d</sup>-region antigens, the helper molecules also have specificity for the public cross-reactive specificities, such as H-2.3 and H-2.49 or their cellular targeted equivalents (21). Because these public determinants are associated with both the K<sup>k</sup> and D<sup>k</sup>-encoded molecules, the B10.BR strain would have a greater number of such determinants than has either the B10.A(2R) or B6.H-2<sup>ab</sup> strain (21). The concentration of determinants on cell surfaces may, therefore, be reflected in their immunogenicity or potential as cytolytic effector cell targets.

**Assessment of MHC Region Cross-Reactivities of B10-Anti-18R-primed Cytotoxic Cells.** The cross-reactivity observed between the B10.BR strain K<sup>k</sup> and D<sup>k</sup> antigens and those of the 18R strain D<sup>d</sup> was corroborated in killer cell assays with C57BL/10 LNC sensitized against gamma-irradiated 18R spleen cells. Blast cells from the B10.BR, B10.A(2R), B6.TL<sup>a</sup> strains, the B10.D2-H-2<sup>dm1</sup> mutant, and the DBA/2 (H-2<sup>d</sup>)-derived P815-X2 mastocytoma tumor cells served as <sup>51</sup>Cr-labeled target cells to assess specificity and cross-reactivity of killing. Several conclusions can be drawn concerning the antigenic targets of the B10 anti-18R killer cells. First, the highest degree of killing was obtained against the H-2D<sup>d</sup>-bearing P815-X2 target cells (Fig. 7). Second,
DM1 mutant-derived cells were readily killed and thus must bear adequate numbers of determinants in common with 18R to serve as targets. Third, little killer activity was directed against the Qa-1a, TLaa determinants, as evidenced by the data obtained from the B6.TLaa recombinant (Fig 7). Fourth, a substantially greater degree of killing was observed when public, cross-reactive antigens were expressed by both the Kk and Dk molecules of the B10.BR strains, as contrasted with only those of the Kk molecules of the B10.A(2R) strain (Fig. 7). The latter data, therefore, coincide with the degree of cross-reactivity observed when SgHF was used to support the generation of killer cells with B10.BR vs. B10.A(2R) fixed stimulator cells (Figs. 5 and 6). The data presented in Figs. 3–7 suggest, therefore, (a) that the early phase SgHF contain antigen-specific helper activity specific for both private (H-2.4) and public (H-2.3,49) cross-reactive determinants encoded within the H-2Dk region; (b) that the target antigens are not encoded within the Qa-1, TLak region; and (c) that the second phase of skin graft-induced helper factor production is somewhat nonspecific in nature. These conclusions were substantiated in further specificity studies as well as in assays for IL-2 activity.

Genetic Mapping of the Antigenic Specificity of Early Phase SgHF. Various H-2 recombinant and mutant lines of mice and either 6- or 8-d SgHF were used in these studies (Table I). The Qa-2 and Qa-1a, TLaa regions were eliminated from consideration in these mapping studies, as the B10 and 18R lines do not differ at the Qa-2 region, and the Qa-1a, TLaa region determined antigens did not serve as targets for 18R primed B10 killer cells (B6.TLaa data, Fig. 7).

Recombinant Lines. SgHF consistently supported the enhanced generation of killer cells with glutaraldehyde-treated cells from the 7R-stimulating donors (Fig. 8, right), whereas virtually no support of killer cell development was consistently observed with the ozl-stimulating cells (Fig. 8, center). Substantial support was provided by SgHF for 18R-stimulated killer cell generation (Fig. 8, left). The B10.S(7R) recombinant line bears the H-2Dk, Lk molecules in common with the 18R line. The B6.H-2ozl recombinant line differs from the 18R donor and B10 recipient lines only in its Dk, Lk.
molecules. These data, therefore, support the earlier suggestion that we could, indeed obtain antigen-specific soluble helper activity during H-2 antigen priming by skin allografts. Furthermore, the antigen specificity of the soluble help can be mapped to the H-2D\(d\) region.

Mutant Lines. A number of distinct cell surface molecules result from the genetic information contained within the H-2D region (22–25). Mice bearing mutations in the H-2D-region complex have been most instrumental in the description of these cell surface molecules. We undertook, therefore, studies with selected H-2D-region mutant lines of mice to further refine the antigenic specificity of our soluble SgHF activity.

The B10.D2-H-2\(d^{dm1}\) (DM1) and BALB/c-H-2\(d^{dm2}\) (DM2) mutant lines were used for these studies (Table I). The DM1 mutant, apparently, is a result of a more complex mutation than initially perceived, but a consistent finding is that the private H-2.4 specificity is expressed in substantially lower concentrations than is detectable by serological means on the parental B10.D2 line (H-2\(d\)) (26–29). Recent studies (28) suggest that the DM1 mutant also expresses lower levels of public antigens within both the D and L molecules. The DM1 mutant line, however, does contain sufficient D-region cell surface antigens to serve as a target for B10 anti-18R generated cytotoxic effector cells (Fig. 7). The BALB/c-H-2\(d^{dm2}\) does not express the H-2L\(d\) (or H-2R\(d\)) defined specificities, but its H-2D\(d\) molecule is expressed in normal quantities (23, 25).

Only minimum support for the generation of B10 anti-DM1 cytotoxic effector cells was observed with SgHF, as contrasted with the nonspecific HFS (Fig. 9 B) when the sensitized cells were assayed on the H-2\(d\)-bearing P815-X2 tumor cells. The same SgHF readily supported the development of effector cells when the B10 responders were primed with 18R-fixed cells (Fig. 9 A). In contrast, we found that SgHF supported DM2-primed generation of killer cells when assayed against either DM2 or P815-X2 targets (Fig. 10 A and C). We considered the possibility that SgHF support for priming by the mutant lines might have been MHC-I-region restricted for the stimulating cell. Thus, the generation of cytotoxic effector cells supported by SgHF
Fig. 9. H-2 antigenic specificity of early phase, 8-d B10 anti-18R-induced SgHF. The H-2\(^{d}\)-bearing P815-X2 tumor cells served as target cells for these assays. Glutaraldehyde-fixed cells served as stimulators in C57BL/10 LNC cultures supplemented with medium alone (●), 20% 8-d SgHF (○), or nonspecific HFS (▲).

Fig. 10. H-2D\(^{d}\) antigenic specificity of early phase, 6-d B10 anti-18R-induced SgHF. Glutaraldehyde-fixed cells from the DM2 line (A, C), the BALB/c parental line (B), and (B6 × DM2)\(F_1\) hybrids (D) served as stimulators of C57BL/10 cytotoxic effector cell generation. The cultures were supplemented with fresh medium alone (●), 20% 6-d SgHF (○), or nonspecific HFS (▲).
was compared with that supported by nonspecific HFS upon priming with fixed cells from the parental lines as well. The cytotoxic effector cell activity generated with the DM2 was substantially greater than that observed upon addition of either SgHF or HFS to the BALB/c parental line (H-2^d) (Fig. 10 B). Furthermore, being concerned with other possible restriction elements of the DM2 stimulator strain because the BALB/c strain differs from B10.A(18R) at many additional loci, we tested F1 hybrid fixed cells from a mating of C57BL/6 and DM2 (H-2^b/H-2^am). SgHF again readily supported the enhanced generation of cytotoxic effector cells (Fig. 10 D). SgHF also can support the generation of killer cells upon priming with the native H-2D^d-bearing B10.D2 line. Both the DM1 mutant cells and P815-X2 can serve as targets for this killing activity (Fig. 11 A and C). Again, little cytotoxic effector cell generation was supported by SgHF with DM1 priming cells (Fig. 11 B and D). This is in direct contrast with the support of helping activity provided by the nonspecific HFS (Fig 11 B and D). It should be noted that the mutational events giving rise to DM1 might have resulted in new target specificities for cellular responses, particularly because the B10.D2 parental line rejects DM1 skin grafts and new serologically detectable determinants have been found (3, 28). The DM1 line also differs from the B10 responder line for the K^d target antigens as well as the gain mutant specificities. The fact that the SgHF provided little or no support for these new antigenic specificities

![Graphs showing the H-2 antigenic specificity of early phase, 8-d B10 anti-18R-induced SgHF. Glutaraldehyde-fixed B10.D2 or DM1 stimulators were cultured with C57BL/10 LNC and supplemented with medium alone (○), 20% 8-d SgHF (□), or nonspecific HFS (△). Each set of cultures was assayed against both P815-X2 and DM1 Con-A blast cells.]

Fig. 11. H-2 antigenic specificity of early phase, 8-d B10 anti-18R-induced SgHF. Glutaraldehyde-fixed B10.D2 or DM1 stimulators were cultured with C57BL/10 LNC and supplemented with medium alone (○), 20% 8-d SgHF (□), or nonspecific HFS (△). Each set of cultures was assayed against both P815-X2 and DM1 Con-A blast cells.
or for those of the H-2K<sup>d</sup> region presented by DM<sup>1</sup>-stimulating cells also attests to its antigenic specificity, particularly for the D<sup>d</sup> region antigens.

These data, therefore, support the conclusion that skin allograft priming results in the production of antigen-specific helper factor activity and, furthermore, map the specificity determinants on the H-2D<sup>d</sup> molecule. The antigen specific helper factor activities are produced and most readily detected as such during the first, early phase of the response. The later or second phase of factor production is complicated by the production of nonspecific factors, including IL-2.

**Kinetics of IL-2 Production after Skin Allograft Priming.** IL-2-containing supernatants obtained from the Con A-inducible, IL-2-producing T cell hybridoma FS6-14.13 served as the positive control in an OVA-specific blast cell proliferative assay (18, 19). C57BL/10 supernatants obtained on the various days after allograft priming (4D-19D), after MLR sensitization (HFS) and Con A activation (Con-A TCGF), were added to 1 × 10<sup>4</sup> blast cells. The ability of these supernatants to maintain proliferation of blast cells was evaluated 4 d later through their incorporation of [3H]thymidine. Nonspecific HFS, Con-A-induced TCGF (IL-2), and hybridoma produced IL-2 all supported OVA-primed blast cell proliferation (Fig. 12). IL-2 activity was not detected in the early 4-, 6-, and 8-d SgHF, but substantial activity was detected in the 10-, 12-, and 19-d SgHF. Thus, the kinetics of IL-2 secretion during allograft priming lend further support to our conclusions concerning the biphasic nature of helper production, with soluble antigen-specific helper activity release being an early phase event followed by the stimulation of nonspecific helper factors and IL-2 release.

**Discussion**

We demonstrated that soluble helper molecules specific for H-2 antigens are produced during the development of immune responses to class I MHC molecules presented by skin allografts. The H-2 antigen-specific helper molecules are generated as a relatively early event during antigen priming. We suggest on the basis of our kinetic data that the production of H-2 antigen-specific helper molecules may precede priming of pre-effector cells. The antigen-specific helper molecules plus antigen would subsequently trigger the effector cell differentiation pathway(s). Neither the antigen alone nor the antigen-specific soluble helper molecules alone are sufficient
for the generation of cytotoxic effector cells in our assays in vitro. The clonal expansion or continued rapid expansion of these primed pre-effector cells may be further mediated by nonspecific helper factors or growth factors such as IL-2. The T cells producing such nonspecific growth signals may be quite distinct from those producing the antigen-specific helper molecules or may represent a different phase of T helper cell maturation. The differentiation of helper T cell functions was particularly evidenced in our kinetic data, as we found nonspecific helper factor production at a later phase after H-2-disparate allograft priming. These latter phase helper or amplifier T cells may eventually become memory cells. Thus, upon restimulation by appropriate antigen, such memory T cells would release nonspecific helper factors.

The production of nonspecific helper factors by cloned antigen-specific helper T cells has been well documented (14–16). In fact, we suggest that the propagation of antigen-specific helper T cell clones on the basis of growth dependence upon IL-2 may result in the specific selection of memory cells rather than selection for primary helper cells. If, indeed, it is only the initially primed helper T cells that produce antigen-specific helper factors, it might be quite difficult to then continuously propagate such cells during this early differentiation phase; hence, the long-term differentiated clones would not be likely to produce the antigen-specific helper molecules but would produce nonspecific factors, as has been observed (14–16).

The existence of memory helper T cells that produce predominantly nonspecific helper factors could also explain the ready detection of nonspecific factors after polyclonal activation of helper T cells with H-2I-region differences in an MLR or with mitogens such as Con A. Our inability to detect antigen specificity of soluble help after MLR priming could be a result of such memory responses after Ia antigen triggering in association with foreign serum borne antigens in the cultures or by syngeneic Ia triggering of an autologous T cell response (30). MLR-derived supernatants would then contain nonspecific helper or amplifier factors that mask the detection of antigen-specific helper molecules. In fact, the identification of antigen-specific helper molecules within the myriad of factors produced after Con A stimulation has recently been documented (31). Such polyclonal activation of memory T cells does not readily occur during skin alloantigen priming within draining lymph nodes. Thus, the identification of soluble antigen-specific helper molecules was greatly facilitated by the absence of other nonspecific factors.

The specificity of the antigen-specific helper molecules induced by allograft priming maps to the H-2D^d molecules presented by the foreign tissue. The SgHF is by no means monoclonal or nonspecific in nature. The recipient’s helper T cells recognize multiple antigenic determinants on the H-2D^d molecules, including H-2.4, H-2.3, and H-2.49 (20, 21). The H-2.3 and H-2.49 antigens are cross-reactive or public specificities, and evidence for both cytotoxic effector cells and later phase helper factors directed against such determinants was provided by the B10.BR and B10.A(2R) strains. The genetic mapping of the helper specificity to the H-2D^d region was accomplished through the use of appropriate recombinant lines of mice such as the 7R, oz1, and B6.TLa^a lines. The precise mapping to the H-2D^d molecule, however, was accomplished with the DM1 and DM2 mutant lines. These mutant lines bear the same H-2 haplotype for the H-2K^d through H-2S^d region and differ only in the H-2D region. Whereas minimal help was provided for responses to DM1 strain cells by early phase SgHF, good support of cytotoxic effector cell generation was provided for DM2
strain cells. Several conclusions can be reached from these data. (a) The early phase SgHF contains little or no nonspecific helper activity, as the SgHF did not support the generation of responses to the H-2K<sup>d</sup> antigens presented by the DM1 strain. (b) Although cellular responses can be generated against the gain or new antigenic specificities that resulted from the mutations within DM1, the B10 anti-18R SgHF again did not support the generation of significant levels of cytotoxic effector cells directed against these "unrelated" determinants. These findings further support the conclusions that little or no nonspecific help is provided by the early phase SgHF. (c) The help provided to DM2 strain cells in contrast to DM1 precisely maps the molecular specificity of SgHF to antigenic determinants of the H-2D<sup>d</sup> base molecule, as the DM2 lacks the H-2R and H-2L molecules (22-25). Thus, we can conclude that helper T cell clones specific for class I MHC antigens do indeed exist and that at least one of their functional roles leads to the secretion of soluble antigen-specific helper molecules. The antigen-specific helper molecules in association with antigen provide a signal for the triggering of effector cell differentiation presumably in vivo as well as in vitro, as we have demonstrated.

Summary

Antigen-specific soluble helper molecules are produced during major histocompatibility complex-disparate allograft priming. Genetic mapping studies with appropriate recombinant and mutant lines of mice have defined the antigen specificities of the soluble helper molecules described here as being directed against the H-2D<sup>d</sup> molecules. The production of antigen-specific helper molecules is a relatively early event after H-2D<sup>d</sup>-region allograft priming. A later phase of factor production near the time of graft rejection also contains nonspecific helper factors and IL-2.

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References

1. Brondz, B. D., I. K. Egorov, and G. I. Drizlikh. 1975. Private specificities of H-2K and H-2D loci as possible selective targets for effector lymphocytes in cell-mediated immunity. J. Exp. Med. 141:11.
2. Schnagl, H. Y., and W. Boyle. 1980. Separate clones of cytotoxic T lymphocytes are generated against private and public H-2 antigens. Transplantation 29:492.
3. Melief, C. J. M., L. P. de Waal, M. Y. van der Meulen, R. W. Melvold, and H. I. Kohn. 1980. Fine specificity of alloimmune cytotoxic T lymphocytes directed against H-2K. A study with K<sup>e</sup> mutants. J. Exp. Med. 151:993.
4. Lindahl, K. F., A. B. Peck, and F. H. Bach. 1975. Specificity of cell-mediated lymholyis for public and private H-2 determinants. Scand. J. Immunol. 4:541.
5. Alter, B. J., and F. H. Bach. 1974. Role of H-2 lymphocyte-defined and serologically defined components in the generation of cytotoxic lymphocytes. J. Exp. Med. 140:1410.
6. Baum, L. L., and L. M. Pilarski. 1978. In vitro generation of antigen-specific helper T cells that collaborate with cytotoxic T-cell precursors. J. Exp. Med. 148:1579.
7. Corley, R. B., and B. Kindred. 1977. In vivo responses of alloreactive lymphocytes
stimulated in vitro. Helper cell activity of MLR-primed lymphocytes. Scand. J. Immunol. 6:923.

8. Kindred, B., and R. B. Corley. 1977. A T cell-replacing factor specific for histocompatibility antigens in mice. Nature (Lond.). 268:531.

9. Plate, J. M. D. 1976. Soluble factors substitute for T-T cell collaboration in generation of T-killer lymphocytes. Nature (Lond.). 260:329.

10. Altman, A., and D. H. Katz. 1980. The induction of autoreactive T lymphocytes by allogeneic effect factor (AEF): relevance to normal pathways of lymphocyte differentiation. Immunol. Rev. 51:3.

11. Plate, J. M. D. 1977. Two signals required for the initiation of killer T-cell differentiation. Cell. Immunol. 32:183.

12. Finke, J. H., C. G. Orosz, and J. R. Battisto. 1977. Splenic T-killer cells can be generated by allogeneic thymic cells in conjunction with assisting factor. Nature (Lond.). 267:353.

13. Delovitch, T. L., J. Watson, R. Battestella, J. S. Harris, J. Shaw, and V. Paetkou. 1981. In vitro analysis of allogeneic lymphocyte interactions. V. Identification and characterization of two components of allogeneic effect factor, one of which displays H-2-restricted helper activity and the other, T cell growth factor activity. J. Exp. Med. 153:107.

14. Augustin, A. A., and A. Coutinho. 1980. Specific T helper cells that activate B cells polyclonally. In vitro enrichment and cooperative function. J. Exp. Med. 151:587.

15. Schreir, M. H., J. Anderson, W. Lernhardt, and F. Melchers. 1980. Antigen-specific T helper cells stimulate H-2 compatible and H-2 incompatible B cell blasts polyclonally. J. Exp. Med. 151:194.

16. Glasebrook, A. L., J. Quintans, L. Eisenberg, and F. W. Fitch. 1981. Alloreactive cloned T cell lines. II. Polyclonal stimulation of B cells by a cloned helper T cell line. J. Immunol. 126:240.

17. Billingham, R. E., and P. B. Medawar. 1951. The technique of free skin grafting in mammals. J. Exp. Biol. 28:385.

18. Schrier, R. D., B. J. Skidmore, J. T. Kurnick, S. N. Goldstine, and J. M. Chiller. 1979. Propagation of antigen specific T cell helper function in vitro. J. Immunol. 123:2525.

19. Harwell, L., B. Skidmore, P. Marrack, and J. Kappler. 1980. Concanavalin A-inducible, interleukin-2-producing T cell hybridoma. J. Exp. Med. 152:893.

20. Klein, J., L. Flaherty, J. L. Vanderberg, and D. C. Shreffler. 1978. H-2 haplotypes, genes, regions, and antigens: first listing. Immunogenetics. 6:469.

21. Klein, J. 1975. In: Biology of the Mouse Histocompatibility-2 Complex. Springer-Verlag, Heidelberg. 126.

22. McKenzie, I. F. C., G. M. Morgan, R. W. Melvold, and H. I. Kohn. 1976. A new H-2 mutant in BALB/cKh that identifies a locus associated with the D-region. Immunogenetics. 4:333.

23. Hansen, T. H., S. E. Cullen, and D. H. Sachs. 1977. Immunohistochemical evidence for an additional H-2 region closely linked to H-2. J. Exp. Med. 145:438.

24. Demant, P., and D. Ivanyi. 1981. Further molecular complexities of H-2-K- and D-region antigens. Nature (Lond.). 290:146.

25. Hansen, T. H., K. Ozato, M. R. Melino, J. E. Coligan, T. J. Kindt, J. J. Jandinski, and D. H. Sachs. 1981. Immunohistochemical evidence in two haplotypes for at least three D region-encoded molecules. D, L, and R. J. Immunol. 126:1713.

26. Dishkant, I. P., A. A. Vedernikov, and I. K. Egorov. 1973. Study of H-2 mutations in mice. III. Serological analysis of the mutation 504 and its derived recombinant haplotypes. Genetika. 9:83.

27. Kohn, H. I., J. Klein, R. W. Melvold, S. G. Nathenson, D. Pious, and D. C. Shreffler. 1978. The first H-2 mutant workshop. Meeting report and summary. Immunogenetics. 7:279.

28. Morgan, G. M., I. F. C. McKenzie, and R. W. Melvold. 1978. The relationship between the H-2 loss mutations of H-2d and H-2b in the mouse. Immunogenetics. 7:247.
29. Klein, J. 1978. H-2 mutations: their genetics and effects on immune functions. *Adv. Immunol.* 26:56.

30. Glimcher, L., D. Longo, R. Schwartz, and I. Green. 1981. Studies on the nature of the stimulating antigen in the murine syngeneic mixed lymphocyte reaction (SMLR). *Fed. Proc.* 40:1061.

31. Kilburn, D. G., and R. Anaka. 1981. Antigen specific helper factors present in the supernatant of Concanavalin A-induced spleen cell cultures. *J. Immunol.* 127:702.