GENETIC CONTROL OF CELL-MEDIATED LYMPHOLYSIS IN MOUSE*

BY DOLORES J. SCHENDEL§ AND FRITZ H. BACH

(From the Departments of Medical Genetics and Surgery, and the Immunobiology Research Center, The University of Wisconsin, Madison, Wisconsin 53706)

The major histocompatibility (H) effects in mouse are primarily associated with genetic disparity in a single region known as H-2, loci of which control expression of H determinants recognized as foreign by a host and that initiate events resulting in rejection. Congenic strains of mice, presumed genetically identical except for H-2, have been useful in understanding the role of this region in the stimulation of allograft responses. Recombinant strains differing for only parts of the H-2 region have helped elucidate the complexity of genetic control by H-2.

Two genetically and functionally separate H antigens have been distinguished which are controlled by loci of H-2. One type of antigen, upon immunization, primarily elicits the formation of cytotoxic and/or agglutinating antibodies which react with cell surface antigens on all lymphocytes and essentially all other tissues. The antibodies are used to type (serologically define) these determinants. Such antigens are designated SD antigens and the H-2 loci, H-2K and H-2D, controlling them as Hsd (histocompatibility SD) loci. The second type of H determinant leads to lymphocyte activation as measured in the mixed leukocyte culture (MLC) test (1, 2). Such determinants are called LD and the H-2 loci controlling their expression Hld (histocompatibility LD) loci. The designations "SD" and "LD" are used to refer to these H differences as a descriptive terminology for distinguishing them.

The differential H effects of LD and SD determinants have been examined in two in vitro test systems used as models for allogeneic interactions. Initial recognition in an allogeneic response leads to enlargement and proliferation of responding cells. This response is, at least in part, assessed in MLC tests. Lymphocytes sensitized in MLC to alloantigens acquire the ability to specifically lyse target cells bearing these same antigens, a response measured in cell-

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Abbreviations used in this paper: CML, cell-mediated lympholysis; H, histocompatibility, [3H]TdR, [3H]thymidine; LD, lymphocyte defined; LPS, lipopolysaccharide; MLC, mixed leukocyte culture; MR, maximum release; PHA, phytohemagglutinin; SD, serologically defined; SR, spontaneous release.
mediated lympholysis (CML) assays (3). Clear distinctions can be drawn between LD and SD determinants in stimulation of these allogeneic responses; this paper presents a detailed analysis of the roles of LD and SD in the generation of CML.

Materials and Methods

Experimental Protocol. Spleen cells from mouse strain A are cultured in one-way MLC with mitomycin C-inactivated spleen cells from strain B. After 4 or 5 days of incubation MLC proliferation is determined by measuring the amount of \(^{3}H\)thymidine (\(^{3}H\)TdR) incorporated by the A dividing cells. The effector capacity of A cells to lyse B target cells is examined in CML by incubating the A cells (sensitized for 4 days in MLC) with sodium \(^{51}C\)-labeled phytohemagglutinin (PHA) blasts from strain B. During the 3-h incubation B cells which are damaged by A effector cells release their \(^{51}C\) into the culture medium.

A positive proliferative response is defined as one in which the counts per minute of \(^{3}H\)TdR incorporated by ABm cultures is significantly greater than counts per minute incorporated by AA~ control cultures. A positive CML response is defined as one in which effector cells sensitized in an ABm MLC cause specific release of \(^{51}C\) from B target cells which is greater than that released from A target cells.

Mice. Mice used in the studies were bred in our colony at the University of Wisconsin; breeding pairs were kindly supplied by Doctors. J. Klein (University of Texas, Dallas, Texas), G. Snell (Jackson Laboratories, Bar Harbor, Maine), and J. Stimpfling, McGlaughlin Research Institute, Columbus Hospital, Great Falls, Mont. Male and female mice between 1 and 6 mo of age were used. With one exception, AQR, all mice were congenic on a C57BL/10 Sn (B10) background, presumably differing for only \(H-2\). Origin of the various recombinant strains has been summarized elsewhere (1). Although the AQR line is not congenic with B10, it carries a considerable portion of the B10 genome having undergone four backcrosses to a B10 parent before being established by intercrossing. Previous studies would indicate that in vitro responses of this strain are primarily governed by \(H-2\) (1).

The \(H-2\) complex may be subdivided into four regions, K, I, S, and D, arranged in that order from left to right on the ninth linkage group. The two outer regions, K and D, contain the Hsd loci, alleles of which determine SD antigens. The I region contains immune response loci; these loci determine the ability of an animal to mount an immune response against certain antigens. Between I and D is a fourth region, S, marked by the Ss locus which controls quantitative levels of a serum protein. One Hid locus has been formally mapped between S and D, and a second locus is presumably located in the I region (2, 4).

In individual strains of mice the four regions may be designated by four letters according to their origins. For instance, the genotype of strain B10.A, which carries the \(H-2^a\) chromosome, is kkdd, it has the K and I regions derived from an \(H-2^k\) chromosome, and the S and D regions derived from an \(H-2^a\) chromosome. Individual strain genotypes are listed in parentheses in tables.

Functional tests are used to define LD and SD phenotypes of particular mouse strains. Two mice are defined as LD different and SD identical if the genetic disparity between them results in significant MLC proliferation, yet serological studies reveal no detectable differences for SD-type antigens. (They may differ by Ia-type antigens which are present on only some lymphoid cells [5].) Alternatively two mice are referred to as SD different if their disparity includes loci that determine different SD specificities and they are not known to be different for the two common Hid loci defined above. It is not possible to define critically the complete LD relationship of such mice since any detectable MLC proliferation could be induced either by SD antigens themselves or by separate LD antigens determined by genes located in the K or D region (marked by the Hsd loci). This point must be kept in mind when one compares animals that have SD differences, these animals differ for entire genetic regions; only part of the genetic material included in such a region may code for SD antigens and it is possible that loci coding for LD antigens may also be present. Thus even though we describe two animals as being "SD different" the reader should be aware of the greater genetic complexity involved.

MLC Test. The MLC system used was a combined modification of the methods of Widmer and Bach (6) and Phillips et al. (7). 40 × 10^4 responding cells were incubated with 60 × 10^4 mitomycin...
C-inactivated stimulating cells in upright culture flasks (Falcon 3012, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). When stimulating cells of two different types were used (i.e., in three-cell cultures) 30 × 10^6 cells of each type were combined to give a total of 60 × 10^6 stimulating cells. Cultures using only one type of stimulating cell were kept at the same cell concentration by adding mitomycin C-inactivated cells syngeneic to the responding cell.

The culture medium used was RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.), supplemented with penicillin, streptomycin, and 5% heat-inactivated (56° C, 30 min) pooled human plasma. After 88-90 h (day 4) or 104-106 h (day 5) of culture, 200 µl aliquots were removed to assess proliferation by [3H]Tdr incorporation. These samples were labeled with 2 µCi [3H]Tdr (sp. act 1.9 Ci/mmol) for 4-6 h. The remaining cell suspension was used in the CML assay.

Heat treatment of stimulating cells was carried out at 45° C for 60 min. Cells were washed once in culture medium after the heat treatment.

CML Assay. Preparation of PHA target cells and the CML assay were carried out according to the method previously described (8). Lipopolysaccharide (LPS) targets were prepared by incubating spleen cells (5 × 10^6 cells/ml) in 3-ml aliquots in RPMI 1640 with 5% heat-inactivated human plasma. 48 h before use they were stimulated with 50 µg/ml of LPS (Escherichia coli) (Difco Laboratories, Detroit, Mich.). MLC blasts were obtained from cultures set up according to the micromethod of Widmer and Bach (6); they were used after 3 days of culture. Normal fresh target cells were prepared from lymph node cells suspensions on the day of the CML assay.

The maximum release (MR) value of each target cell preparation was measured by determining the amount of ^51Cr released into the supernatant fluid by 1 × 10^4 target cells after freeze-thawing three times. Spontaneous release (SR) of ^51Cr from the same number of target cells, incubated for 3 h with 1 × 10^4 freshly prepared lymph node cells, was also measured. The percent specific chromium release was calculated according to the formula: (experimental release − SR)/(MR − SR) × 100. All assays were done in triplicate.

Results and Discussion

Initial studies concerning in vitro MLC and CML responses after stimulation by LD and SD determinants revealed several differences. It was observed that stimulation by LD differences alone produced strong proliferative responses whereas stimulation by SD regions did not lead to activation, or did so only weakly (1, 2). LD differences, with one exception (9), did not generate detectable cytotoxic activity and did not serve as good targets for lysis; while SD differences were important in generation of cytotoxic potential and were recognized by effector cells as targets (8, 10, 11) or served as markers for the actual target detected in CML (9, 12). Cases were noted where stimulation by either LD or SD determinants alone failed to produce detectable cytotoxic activity, but sensitization to both LD and SD at the same time led to positive CML reactions (8, 12).

The Role of LD Determinants as Targets for CML. As previously reported we have examined CML responses of strain pairs that are LD different and SD identical (8). MLC proliferation is observed but cytotoxic responses are negative.

Several factors must be considered when interpreting the failure to obtain CML in combinations differing for LD. A number of technical difficulties may limit detection of cytotoxic activity. Objections have been raised concerning suitability of PHA blasts as targets for lysis directed toward LD determinants. We have utilized other types of target cells in CML to determine whether they are more sensitive for finding LD destruction. Table I compares CML on four different target populations. PHA blasts were used as monitors for the standard CML technique. Spleen cells were stimulated with LPS to produce a blast
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Table I

Comparison of Various Types of Target Cells

| Effector combination | Target cell | PHA-M | LPS | MLC | Normal |
|----------------------|-------------|-------|-----|-----|--------|
| AQR \( + B_{10} \cdot T(6R)_{m} \) \( (qkdd) \) (qqqd) | AQR \( -0.78 \pm 4.1 \) \( -9.4 \pm 3.1 \) \( -1.1 \pm 1.8 \) \( -7.4 \pm 6.1 \) | B10.T(6R) \( 3.4 \pm 3.4 \) \( 8.6 \pm 4.2 \) \( 7.1 \pm 3.6 \) \( -1.4 \pm 7.8 \) | B10.A \( -3.1 \pm 3.7 \) \( -3.4 \pm 4.6 \) \( -0.07 \pm 3.1 \) \( -6.9 \pm 3.6 \) | |
| AQR \( + B_{10} \cdot A_{m} \) \( (qkdd) \) (kddd) | AQR \( -10.4 \pm 3.6 \) \( -16.8 \pm 4.4 \) \( -1.9 \pm 1.4 \) \( -9.8 \pm 4.9 \) | B10.T(6R) \( -1.6 \pm 4.5 \) \( -10.4 \pm 4.7 \) \( -5.1 \pm 2.6 \) \( -7.7 \pm 7.4 \) | B10.A \( 69.3 \pm 8.1 \) \( 54.8 \pm 4.2 \) \( 30.5 \pm 7.1 \) \( 11.7 \pm 3.7 \) | |

The data are expressed as % CML (mean ± SD) obtained after \( 1 \times 10^{5} \) effector cells were incubated with \( 1 \times 10^{6} \) target cells for 3 h.

A population primarily of B-cell type; we wished to examine such a target because of reports that LD determinants may be preferentially expressed on B cells (5). MLC blasts were prepared in an attempt to clonally expand the population of cells recognizing one LD determinant thereby possibly expanding those cells which express LD determinants on their surface as well (13); these cells also eliminated potential effects due to alternations in surface antigens caused by nonspecific stimulation. Finally untreated lymph node cells were also examined; we know that LD is expressed on these cells in such a manner as to cause MLC (D. J. Schendel, unpublished observations).

Essentially identical patterns of response are noted on the four targets. In AQR plus B10.T(6R)\(_{m}\), in which there is sensitization to only LD differences, there is no significant, specific lysis of B10.T(6R) targets. Thus the pattern of cytotoxic reactivity to LD determinants is not altered by substituting other types of target cells. AQR plus B10.A\(_{m}\) produces effector cells that are cytolytic to B10.A targets regardless of their manner of preparation. PHA and LPS blasts show similar levels of susceptibility to lysis while MLC blasts and normal untreated targets show lower levels of destruction. PHA blast cells are more susceptible to lysis than normal small lymphocytes; fresh cells, of course, are small lymphocytes and MLC blast preparations contain considerable numbers of nonblast cells. This difference most likely accounts for the various levels of lysis observed.

Relatively high levels of spontaneous release of \(^{51}\)Cr label from target cells and the ratio of effectors to targets required to measure CML could contribute to an inability to detect relatively weak cytotoxic responses with LD different combinations. On occasion in single experiments we have observed low levels of cytotoxicity (3.1–10.4%) when AQR plus B10.T(6R)\(_{m}\) effector cells are incubated with B10.T(6R) targets. This may indicate that LD can serve as a poor target in CML, however this activity has not been consistently reproduced.

In support of possible low level CML on LD one significant trend has been noted in repeated experiments testing the effector capacity of AQR plus B10.T(6R)\(_{m}\) on various target cells. In 12 of 15 separate experiments the mean percent CML measured on B10.T(6R) targets was greater than that observed on AQR targets, syngeneic to the responding cell, and in 13 of 15 experiments the lysis of B10.T(6R) targets was greater than that of unrelated B10.A targets. These values are significantly different from random chance (chi-square) with \( P \)
values of <0.02 and <0.005, respectively, and may indicate detection of a very weak response directed at either LD determinants or other weak non-SD targets. Detection of weak but significant CML responses with the B10.A(4R) plus B10.A(2R) or B10.A(1R) combinations (LD different, SD identical) has been observed.²

It must be stressed that the level of lysis of B10.T(6R) targets is extremely low in comparison to that observed in other allogeneic combinations where lysis is directed against SD determinants. A compilation of data obtained from 20 separate experiments is presented in Table II. In AQR plus B10.T(6R)m the mean percent CML is -4.8%, and in AQR plus B10.T(6R)m plus B10.Am where sensitization to both LD and SD is possible an average of only 1.1% lysis is obtained on the LD target—in sharp contrast to an average of 60.2% noted on the SD-bearing target cell.

**Table II**

Summary of 20 Experiments Testing Cytotoxic Activity After Stimulation by LD Alone or LD + SD

| Effector combination | CML (mean ± SD) |
|----------------------|-----------------|
|                      | AQR     | B10.T(6R) | B10.A |
| AQR + B10.T(6R)m     | -12.5 ± 13.8 | -4.8 ± 10.0 | -9.5 ± 10.5 |
| AQR + B10.T(6R)m + B10.Am | -8.0 ± 13.9 | 1.1 ± 14.0 | 60.2 ± 33.5 |

These results indicate a sharp disparity between LD-different combinations and other allogeneic combinations as tested in CML. With currently available techniques for generating effector cells and possible target cells for measuring lysis, any cytotoxic activity in LD combinations is at least an order of magnitude less than that observed when SD-region differences are also used for sensitization and as targets. The results however do not rule out the possibility that LD can stimulate strong cytotoxic responses both in vivo and in vitro, and given more sensitive techniques such responses will eventually be measured.

Finally, note should be made of one discrepancy found in this pattern of cytotoxic responses of LD-different combinations; one LD-different, SD-identical strain combination does respond differently. C57BL/6-H(z1) shows strong, reciprocal cytotoxic responses (9). This dichotomy may be explained by one of several possibilities which have been discussed in the past (9, 12).

**Interaction of LD and SD in the Generation of CML.** Collaboration between LD and SD in generating CML is most easily studied in three-cell experiments where the determinants are presented on separate stimulating cells. An example of such a three-cell experiment is presented in Table III. B10.T(6R) is the responding population and LD sensitization is provided by AQRm cells. B10.Gm

²Peck, A., and F. Bach. 1974. Mouse cell-mediated lympholysis assay in serum-free and mouse serum-supplemented media: culture conditions and genetic factors. *Scand. J. Immunol.* In press.
Table III

A Three-Cell Experiment

| Effector combination | Genetic stimulation | MLC (mean ± SD) | CML (mean ± SD) |
|----------------------|---------------------|----------------|-----------------|
|                      |                     |                | AQR  | B10.T(6R) | B10.G |
| B10.T(6R) + AQR<sub>m</sub> (qqqd) | LD                 | 16,817 ± 763   | -3.1 ± 2.2 | -3.9 ± 0.91 |
| B10.T(6R) + B10.G<sub>m</sub> (qkdd) | SD                 | 5,852 ± 480    | -1.4 ± 2.0 | 4.3 ± 1.5   |
| B10.T(6R) + AQR<sub>r</sub> | LD + SD            | 24,334 ± 508   | -0.48 ± 2.6 | -1.9 ± 1.9  | 21.4 ± 2.2 |
| B10.T(6R) + B10.T(6R)<sub>m</sub> | —                  | 2,473 ± 152    | -2.2 ± 1.4 |

This experiment provides an example of a situation in which sensitization by either LD or SD antigens alone does not stimulate strong cytotoxic potential, but together they collaborate to generate specific CML which is directed towards SD targets. This three-cell experiment, together with a similar experiment previously described (12), demonstrate that LD differences collaborate with SD differences due to either H-2K or H-2D genetic disparity to produce cytotoxic effector cells.

Initial studies failed to show CML with many SD combinations differing at only K or D. Using altered culture techniques it has now been possible to obtain CML effectors in most SD combinations. Even in these situations where stimulation by SD alone can lead to CML, a collaborative effect with stimulation by both LD and SD during the MLC sensitization phase produces an enhanced CML response compared to that detected when only SD differences are used for stimulation. Fig. 1 illustrates the cytotoxic potential of cultures stimulated by SD region differences alone (AQR plus B10.A<sub>m</sub>) and those stimulated by both LD and SD differences [AQR plus B10.T(6R)<sub>m</sub> plus B10.A<sub>m</sub>]. Data from AQR plus B10.T(6R)<sub>m</sub> cultures (LD alone) is not presented as no significant CML response is detected. A linear relationship between the log of the number of effector cells and percent CML is observed in both cases. Because the slopes of the two lines are parallel a comparison of activities of the two cytotoxic populations can be made.

Table IV summarizes comparisons between amounts of CML when SD alone and LD plus SD stimuli are present in MLC. At any ratio, effector cells generated by stimulation with LD plus SD differences cause approximately 20% more lysis of SD targets. The number of effectors required to cause 50% lysis of a given number of targets is 4.2-fold less in LD-enhanced cultures compared to the
**Table IV**

*Cytotoxic Activity of Effector Populations Sensitized to SD Determinants Alone and to (LD + SD) Determinants*

| Effector combination | Genetic stimulation | Target | CML* | No. effectors - 50% lysis | No. effectors/culture | No. LU$\ddagger$/culture |
|----------------------|---------------------|--------|------|--------------------------|----------------------|-------------------------|
| AQR + B10.Am         | SD                  | B10.A  | 44   | 4.2 x 10$^3$             | 4.5 x 10$^4$         | 10.7                    |
| (qkdd) (kkdd)        |                     |        |      |                          |                      |                         |
| AQR + B10.Am         | LD + SD             | B10.A  | 66   | 1.0 x 10$^3$             | 8.1 x 10$^4$         | 81.0                    |
| + B10.T(6R)m         |                     |        |      |                          |                      |                         |
| (qqqd)               |                     |        |      |                          |                      |                         |

* The % CML indicated is that obtained when 25 x 10$^4$ effector cells are incubated with 1 x 10$^4$ B10.A target cells.

† Lytic units (LU) are calculated according to the formula (no. effectors/culture)/(no. effectors - 50% lysis).

cultures sensitized to SD alone. Not only is this specific activity of effector populations different in the two cultures, but also there is a twofold difference in the number of effector cells recovered per culture. Using the preceding two values one can calculate the number of lytic units obtained per culture by dividing the number of effector cells recovered per culture by the number of effector cells causing 50% lysis. When these values are determined for both effector populations in eight-fold increase is observed in effectors activated by LD plus SD compared to SD stimulation alone.

In evaluating the role of LD enhancement of cytotoxicity one possibility to consider was that equally strong proliferation and cytotoxicity could occur with stimulation by SD alone and stimulation by LD plus SD but peak responses might occur at different times. This possibility was tested in kinetic studies with AQR-6R-B10.A. Fig. 2 a and b show that this is not the case. Highest MLC pro-
liferation (Fig. 2 a) is seen on day 4 in both cultures. Cytotoxic responses of both effector populations peak on day 5, 24 h after the MLC peak activity (Fig. 2 b). By day 6, CML in cultures sensitized to SD alone is falling off as it is in cultures stimulated by LD plus SD. At no time do responses of the SD-different combination reach the peak levels of response seen with stimulation by both LD plus SD.

Is collaboration by LD an essential event or only a helper effect? Examples have been cited where neither LD nor SD alone stimulate CML but combined sensitization does; such results would indicate an obligate need for LD. However, stimulation by SD-region differences alone can also produce CML responses under some conditions (Fig. 1). It must be emphasized again that when we speak of two strains having SD differences we are speaking of strains that differ for entire SD regions, and certainly only part of the genetic material contained in such a region is coding for SD antigens. Clearly, SD-region differences alone are not totally incapable of activating MLC proliferation (1). The SD regions themselves may contain quantitatively weaker Hld loci that can interact with SD antigens of the same region to produce CML. We have obtained evidence from one series of experiments which provide data consistent with the hypothesis that there are in fact LD-like determinants in the K region of B10.A which are recognized by AQR and collaborate with SD antigens to produce CML.

In human CML studies Eijsvoogel et al. demonstrated that heat treatment of an allogeneic-stimulating cell destroyed its ability to cause strong MLC proliferation but left its ability to sensitize for CML, provided an LD difference was supplied by another nonheat-treated cell (14). In addition, heat treatment has been shown to have no effect upon the ability of cells to absorb anti-SD antibody. These results indicate that heat treatment affects LD but leaves SD unaffected. We have studied effects of heat treatment in the three-cell system. Particularly we wished to focus upon the question of whether AQR and B10.A (SD-region different) have weak LD differences located in the SD region which collaborate to produce CML effectors.

The rationale of the experiment was to use heat treatment to damage any LD differences on the B10.A-stimulating cell while leaving SD antigens intact. One
could then determine whether SD antigens themselves produce CML effectors. Table V shows results of such a heat treatment experiment. The first set of data shows control values for the normal experimental system using mitomycin C to inactivate stimulating cells. The SD combination under these conditions gives positive proliferation and cytotoxicity, and CML is enhanced in the three-cell combination where both LD and SD are used as stimuli. When heat treatment (Δ) is substituted for mitomycin C inactivation essentially all CML activity is lost. The last two lines show responses when both treatments are combined. When the LD-stimulating cell is heat treated and the SD-stimulating cell is inactivated with mitomycin C, CML is not significantly different from that seen with SD stimulation alone in the normal system (25% vs. 22%). More interestingly though when the SD-stimulating cell is heat treated and the LD-stimulating cell is mitomycin C inactivated, CML is as high as that seen in normal three-

| Effector combination | MLC activation* (mean ± SD) | % CML‡ (mean ± SD) |
|----------------------|-----------------------------|-------------------|
| AQR + B10.Am         | 4,113 ± 190                 | 21.8 ± 2.2        |
| AQR + B10.Am + B10.T(6R)m | 8,292 ± 309 | 40.4 ± 2.8        |
| AQR + B10.AΔ         | 4,332 ± 89                  | 3.1 ± 1.9         |
| AQR + B10.AΔ + B10.T(6R)Δ | 5,771 ± 301 | -3.2 ± 2.6        |
| AQR + B10.Am + B10.T(6R)Δ | 8,771 ± 287 | 25.3 ± 3.5        |
| AQR + B10.AΔ + B10.T(6R)Δ | 17,375 ± 426 | 42.7 ± 2.9        |

* The AQR + AQRΔ cultures gave 2,926 ± 254 cpm and AQR + AQRΔ gave 2,316 ± 107 cpm. ‡ The % CML of the B10.A target cell is presented. Δ, 45°C for 60 min.

Table V
The Effect of Heat Treatment of Stimulating Cells on the Generation of Cytotoxic Responses

(43% vs. 40%). Heat treatment abolishes cytotoxicity of populations stimulated by SD alone or by both LD and SD; but CML can be restored by adding back a normal LD stimulus on a mitomycin C-treated cell. This indicates that in AQR plus B10.AΔ the SD-region difference is still expressed in such a manner that it can sensitize for target recognition, but it is critically affected in its ability to produce cytotoxic effector cells without an added normal LD stimulus. Thus heat treatment may actually be damaging an LD difference which is controlled by a locus closely linked to the H-2K locus of B10.A and located within the SD (K) region. If this interpretation is correct it would suggest that collaboration between LD and SD is essential in generation of CML. Alternatively one could argue that heat treatment affects only one molecule (i.e., the SD antigen) but molecular requirements for proliferation and cytotoxicity are different.
Heat treatment does not in all cases totally eliminate proliferation. However the lower percentage of cells recovered from cultures having only heat-treated stimulating cells indicates that proliferation is being affected, and the degree of this effect may not be totally apparent when proliferation is measured beyond the time of peak MLC activity. In this experiment both MLC proliferation and CML were assessed on day 5.

General Discussion

$H-2$ contains genetically distinct loci which control at least two types of H antigens, the LD and SD components. In addition to being able to genetically dissect these determinants certain functional distinctions between them can also be made.

Genetic separability of LD and SD is based on classical genetic recombination; the functional dichotomy is less clearly established but seems well taken on several grounds. Primarily $H-2$ LD-type differences lead to strong proliferation of T lymphocytes in MLC while "serving" as poor targets in CML; $H-2$ SD-type differences are relatively weak in causing proliferation but serve as excellent targets. Since we cannot differentiate experimentally between the ability of LD or SD to initiate cellular events which lead to cytotoxicity versus their serving as targets in CML we will simply speak of them in terms of their serving as targets. It is difficult to evaluate whether SD antigens of $H-2K$ and $H-2D$ lead to proliferation. Cells of mouse strains differing by either K or D stimulate a proliferative response, although on the average this response is less than that evoked by differences of the strong LD locus. The heat treatment experiment presented in this paper suggests that in addition to the $H-2K$ or $H-2D$ locus there are LD-like stimuli in these regions, the phenotypic expression of which is altered by heat treatment. If such an interpretation is correct, the SD antigens themselves are either not stimulatory at all or only very weakly so in terms of leading to proliferation.

Contrary to the findings in the past, the data analyzed here suggests that LD differences may serve as very weak targets in CML; however they do so to a much lesser extent than do SD-type differences. It is not clear that SD antigens are the targets for cytotoxic lymphocytes; SD antigens may serve as markers for loci closely linked to those determining the SD antigens, the products of the latter loci being the targets in CML.

It has been argued in the past that the reason that LD differences are not good targets in cytotoxicity may be that they are not well expressed on PHA blasts. We have tested a series of targets to determine whether LD antigens could be detected in CML. Results with these cells argue that it is not simply PHA stimulation which explains the relatively poor target nature of LD. Experiments by Alter and Bach (15) using normal lymph node cells as targets in CML also failed to show strong cytotoxic activity against LD differences; although such cells function as excellent targets in combinations differing by both LD and SD. These experiments taken together once again support the general dichotomy between LD and SD as targets in CML.

Two mutant mouse strains have been tested that show unusual patterns in the functional dichotomy of LD and SD. One mutant, discovered by Bailey et al., carries a spontaneous
mutation in the \( H-2 \) chromosome of the C57BL/6By mouse (16) which leads to reciprocal skin graft rejection; nevertheless, upon reciprocal immunization no antisera defining SD-type antigens have been evoked. Cells of these strains also stimulate strongly in MLC and lead to excellent CML (9). This could be interpreted as LD differences serving as excellent CML targets. Similarly the mutant of Egorov, a spontaneous mutation apparently affecting the \( H-2D \) locus product of the \( H-d^a \) chromosome, evokes both MLC reactivity and skin graft rejection with the parental strain (17). This might be taken as pure SD-type differences causing strong proliferation. One must be cautious in interpreting these mutations as simple events affecting only LD or SD. It is conceivable that these mutations, while spontaneous, are not point mutations but represent something more complex which affects multiple closely linked loci. Alternatively LD- and SD-phenotypic products may interact at the molecular level. Such an interaction could lead to allosteric phenomena whereby a change in configuration of one molecule could lead to a configurational change in the second molecule. Further if differences between LD and SD are quantitative, simple mutational events could strongly effect their phenotypic expression. These mutant strains are thus not of great value in critically dissecting the relative roles of LD and SD.

While the functional distinction between \( H-2 \) LD and SD components seems strongly supported by these many experiments, it is not necessary to think of them as being qualitatively different at the molecular level. It is conceivable that surface density, distribution, or rates of shedding may all affect relative physiological roles of these components in the reactions we are studying.

The nature of the LD-SD interaction can be analyzed at two levels—one can consider the interaction at the genetic level, or at the cellular level. The addition of an LD stimulus, increases the CML response to the specific SD target. Whether the LD stimulus is essential to allow development of CML is a question which only can be related to a particular experimental system. Given the heat treatment experiment presented in this paper, we would argue that, under the present experimental conditions and allowing the interpretation that the heat treatment damaged an LD-type stimulus in the K region, the interaction may well be essential.

The LD-SD interaction may involve two subpopulations of lymphocytes or a single responding population. Given two separate responding populations, we might postulate that one population, a proliferating helper cell, responds primarily to LD-type stimuli; this response may then enable a second population, the eventual cytotoxic lymphocyte, to react to the appropriate target (8). Whether it is something directly related to proliferation of the first population which is essential to allow the second population to respond or whether the LD stimulus evokes quite a separate response (which might be distinct from the proliferative events themselves) which is important in the interaction between the two populations is a subject for further experimentation.

The well characterized hapten-carrier system of dual antigenic control of antibody production may serve as a model for analogy. Here two antigenic signals are needed to stimulate separate populations of responding cells and these cells then collaborate to produce the final antibody response.

There appears to be a dichotomy in the hapten-carrier and LD-SD analogy. Hapten-carrier signals must be presented on contiguous molecules while LD and SD determinants
can be presented on separate cells. This difference need not be problematic if we consider the number of cells responding to different antigenic signals. In the hapten-carrier system this number is quite small and therefore contiguous molecules may be important to bring two populations into close proximity to allow interaction; the number of cells responding to an LD signal is quite high and the probability of an LD-responsive cell contacting its collaborative partner would be much greater and overcome the need for an antigen “bridge.”

The response to LD and SD could also be based on a single responding cell population, since the two-cell model is not critically proven. One could either hypothesize that a single responding cell has two types of receptors, one recognizing LD differences the other SD differences. LD recognition and response might be essential before the cell can recognize SD differences and develop into a cytotoxic lymphocyte. Alternatively one might hypothesize, as Cohn has done for antibody production (18) that an associative recognition takes place. The LD-different stimulating cell may recognize the eventual cytotoxic effector cell and give it a signal which permits it to recognize the SD difference.

That proliferation has something to do in this overall process is based on a large body of indirect evidence. In those combinations where weak “LD-type” differences exist, ability to develop a cytotoxic response is consistently linked to finding significant proliferation. We have also found that the Mls locus of Festenstein (10) can substitute for H-2 LD differences in the LD-SD interaction (D. J. Schendel, unpublished observations).

If the import of LD is related to a proliferative stimulus the nature of the signal is more than nonspecific expansion of all cells. The strongest evidence for this comes from the comparison of the cytotoxic potential of cultures stimulated by SD regions alone or those stimulated by both LD and SD. In the latter case not only are more cells recovered from MLC, but also the specific activity of the effector population has changed such that only one-fourth as many cells are required to cause 50% lysis of a given number of target cells. This can not be accounted for if LD merely serves to stimulate all cells to divide; rather there must be an enrichment of the specific effector cells.

Summary

H-2 congenic mouse strains were tested in vitro to investigate the genetic control of cell-mediated lympholysis (CML). Combinations were selected such that differences in various segments of H-2 could be examined for their ability to stimulate production of effector cells and to serve as targets for lysis. Particular emphasis was directed towards understanding the roles of LD and SD.

SD-region differences are important in the sensitization of effector cells and they also function as strong targets for lysis, or as markers for the CML targets. LD differences are also important for sensitization of cytotoxic effector cells, but they serve only as very weak targets for lysis.

Collaboration occurs between LD and SD in generation of CML. The nature of this interaction can be of two types: together LD and SD can produce CML which neither difference alone can stimulate; LD can enhance a CML response stimulated by SD-region differences alone.
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