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Immunology

Identification of a fourth class of proteins linked to the murine major histocompatibility complex

(alloantisera/congeneic strains/two-dimensional electrophoresis)

JOHN J. MONACO AND HUGH O. MCDEVITT

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT **A series of proteins biochemically and genetically distinct from previously defined murine major histocompatibility complex class I and class II antigens is precipitated by a congeneic anti-H-2^d antiserum. Sixteen such proteins have been defined, exhibiting a range of molecular weights (approximately 15,000–30,000) and isoelectric points (pI approximately 4–9). These proteins are not glycosylated, and they are probably not expressed at the cell surface. They are expressed most strongly in normal macrophages and macrophage cell lines and are also found in fibroblasts, B, T, and null cell lines. The genes controlling the expression of these proteins have been tentatively mapped within the *H-2* complex, between the *K* and *I-A* subregions. Three alleles have been defined: mice of the *H-2* haplotypes *b* and *q* possess a "null" allele, i.e., do not express any demonstrable protein product. Mice of the *d* haplotype can be distinguished by their two-dimensional gel pattern from mice of all other positive *H-2* types tested thus far (*a*, *k*, *f*, *s*, and *ja*).**

The major histocompatibility complex (MHC) of the mouse (and of many other species, including man) has been shown to encode three biochemically and functionally distinct classes of antigens (for review, see ref. 1). The class I antigens (H-2K and D, Fig. 1) are 45,000 molecular weight (M_r) glycoproteins which are noncovalently associated at the cell surface with a 12,000 M_r, non-H-2-encoded polypeptide, β_2-microglobulin (2). These molecules are found on all nucleated cells and are the classic serologically defined major transplantation antigens. The class II (Ia) antigens are also two-chain cell surface glycoproteins, composed of a heavy chain of 33,000–34,000 M_r and a light chain of 28,000–29,000 M_r (3–5). These antigens display a more restricted tissue distribution than do class I antigens, being found mainly on B cells, macrophages, and epidermal cells (6). Both the class I and class II antigens are of fundamental importance in the recognition and interaction of the cellular components of the immune system and have been implicated in the regulation of immune responses (7–14).

The class III MHC antigens are encoded in the *S* region of the H-2 complex (Fig. 1) and are high molecular weight serum glycoproteins. These molecules have been shown to be key components of the complement system (15) and are thus also intimately involved in immune regulation as an important effector arm of the humoral immune response.

While examining the expression of class I and II antigens on murine macrophage cell lines, we noticed that an antiserum made in congeneic H-2^b mice against the H-2^d haplotype had reactivity for a series of proteins biochemically and genetically distinct from class I and II antigens. We report here the biochemical properties of these proteins, their linkage to the *H-2* complex, and preliminary genetic mapping data.

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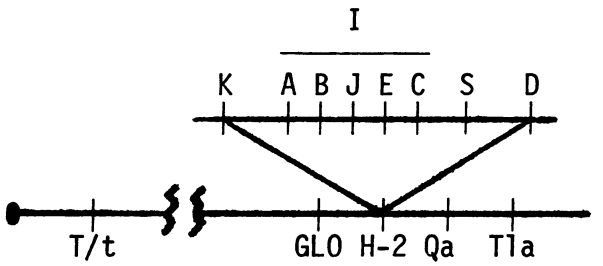

FIG. 1. Genetic map of the H-2 complex on chromosome 17. Centromere is denoted by knob at left.

MATERIALS AND METHODS

Cell Lines. The cell lines used and their descriptions are listed in Table 3. All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Animals. B10.GD mice were the kind gift of P. Jones (Dept. of Biological Sciences, Stanford University), and B10.MBR mice were generously provided by C. S. David (Mayo Clinic, Rochester, MN). All other mice were from our breeding colony at Stanford.

Antisera and Adsorptions. BALB.B anti-BALB/c (anti-H-2^d), A.TH anti-A.TL (anti I^k), and C3H anti-CSW (anti-H-2^b) antisera were produced as described (16). Serum for adsorption was diluted 1:1 with Dulbecco's phosphate-buffered saline containing 0.1% sodium azide and adsorbed twice with an equal volume of pelleted spleen cells for 1 hr on ice.

Radio Labeling and Immunoprecipitation. Cells were labeled in 2 ml of methionine-free RPMI 1640 medium (GIBCO) at 1–2 × 10^7 cells per ml for cell lines or 3–4 × 10^7 cells per ml for normal spleen cells. [^{35}S]Methionine (New England Nuclear, >500 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was added to a final concentration of 250 μCi/ml for cell lines or 500 μCi/ml for spleen. Labeling was allowed to proceed 4–6 hr at 37°C in a humidified atmosphere of 5% CO_2, after which cells were washed and extracted with 0.5% Nonidet P-40 as described by Jones (5). Generally, 100–200 μl of cell lysate was incubated on ice for 25 min with 30–50 μl of antiserum. Then 200 μl of a 10% suspension of fixed *Staphylococcus aureus* (IgGsorb, Enzyme Center, Boston, MA) was added and incubation was continued for another 25 min. Precipitates were then washed and eluted into isoelectric focusing sample buffer (17).

Two-Dimensional Gels. Two-dimensional polyacrylamide gel electrophoresis was carried out as described by Jones *et al.* (17), using nonequilibrium pH gradient electrophoresis for sep-

Abbreviations: MHC, major histocompatibility complex; LMP, low molecular weight proteins.

aration by charge in the first dimension, and $NaDodSO_4$ gel electrophoresis for size separation in the second dimension. For fluorography, stained gels were treated with sodium salicylate, dried, and exposed to Kodak XAR-5 film as described by Chamberlain (18).

RESULTS

Immunoprecipitation from Cell Lines. Anti-H-2^d immunoprecipitates from the DBA/2 and BALB/c-derived macrophage-like cell lines P388D1 and WEHI-3 were analyzed by two-dimensional polyacrylamide gel electrophoresis. The resulting autoradiographs are shown in Fig. 2 *B* and *D*. As expected, the 45,000 M_r H-2K and D products are present in both precipitates. WEHI-3 (but not P388D1) also expresses the α, β, and 31,000 M_r (invariant) chains of Ia antigens. In addition, both immunoprecipitates contain a series of 16 heavily labeled proteins of low molecular weight (approximately 15,000–30,000). A high-titer antiserum directed only against products of the I^k region (A.TH anti-A.TL) does not precipitate these low molecular weight proteins (LMP), although it precipitates Ia antigens from WEHI-3 (Fig. 2 *A* and *C*). In other experiments (not shown) an anti-H-$2D^d$ [(B10.BR × A.SW) F_1 anti-B10.S(7R)] antiserum also failed to recognize the LMP, demonstrating that their precipitation by the anti-H-2^d antiserum is highly specific.

LMP Are Not Viral Proteins. Cell lines often express viral proteins, and H-2 antisera may be contaminated with anti-viral antibody (19–21). Two lines of evidence suggested that the LMP are not virus-related proteins: (*i*) Antisera made specifically against purified murine leukemia virus proteins were unable to precipitate any of the LMP, and (*ii*) no gp69/70 is evident in any of the immunoprecipitates from these cell lines (unpublished observations). It is therefore unlikely that the LMP represent viral proteins. This conclusion is supported by the genetic mapping data shown below.

Adsorption of Antibody Activity by Normal Cells. To determine whether these proteins represented tumor-specific antigens or were also present on normal cells, the anti-H-2^d serum was adsorbed with mouse spleen cells and then tested for precipitating activity on extracts of the WEHI-3 cell line. BALB/c spleen cells were capable of completely adsorbing the precipitating activity, whereas adsorption with BALB.B spleen had no effect (Table 1).

These data indicate that the LMP are present on normal cells and also that the genes regulating their expression are tightly linked to H-2. This linkage was confirmed by using two other pairs of H-2 congeneic mice; B10 (H-2^b) mice are negative, whereas their H-2^d congeneic partner strain, B10.D2, is positive. C3H mice (H-2^k) are also positive, and their H-2^b congeneic partner strain, C3H.SW, is negative (Table 1).

Haplotype Distribution and Genetic Mapping. A haplotype distribution pattern was established on B10 congeneic lines. As can be seen in Table 1, mice of the *b* and *q* haplotypes (B10 and B10.G) are negative, whereas mice of the *d*, *k*, *a*, *f*, *s*, and *ja* haplotypes are positive.

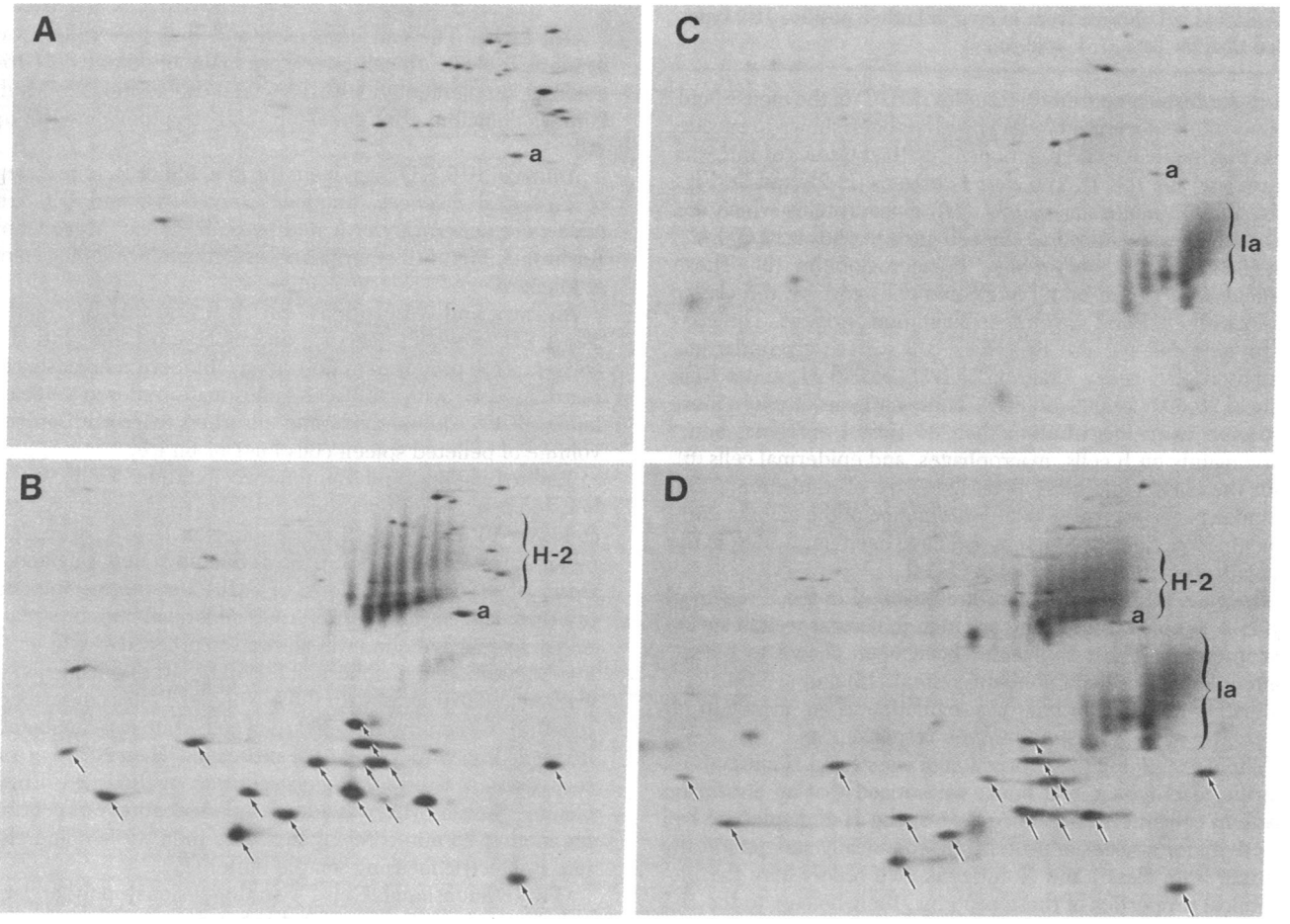

FIG. 2. [^{35}S]Methionine-labeled P388D1 (*A* and *B*) and WEHI-3 (*C* and *D*) cell extracts were precipitated with either anti-I^k (*A* and *C*) or anti-H-2^d (*B* and *D*). Proteins were separated by charge (right to left is acidic to basic) in the horizontal dimension and by size (top to bottom is high to low molecular weight) in the vertical dimension. H-2K and D, Ia α and β chains, and actin (a) are indicated. Arrows indicate the low molecular weight proteins (LMP).

Table 1. LMP haplotype distribution

Strain	H-2 type	Adsorption
BALB/c	*d*	+
BALB.B	*b*	−
BALB/K	*k*	+
C3H	*k*	+
C3H.SW	*b*	−
B10	*b*	−
B10.D2	*d*	+
B10.BR	*k*	+
B10.A	*a*	+
B10.G	*q*	−
B10.M	*f*	+
B10.S	*s*	+
B10.WB	*ja*	+

Results from typing a panel of intra-H-2 recombinant haplotypes, either by adsorption or by direct precipitation (see below), are presented in Table 2. Data from the first six strains are all consistent with a map position to the left of the *I-A* subregion. The crossover event in the B10.AQR strain thus defines the right-hand boundary of the locus controlling LMP expression.

The B10.MBR strain is crucial for determining the left-hand boundary. The *H-2K* locus in this strain is derived from the B10 (H-2^b) parent, whereas the entire *I* region is of *k* haplotype origin (derived from B10.AKM). Assuming a single crossover event in this region (see *Discussion*), the genes governing the expression of LMP lie within the *H-2* complex, between the *K* and *I-A* subregions.

Direct Precipitation from Normal Cells. BALB/c spleen cells were internally labeled with [^{35}S]methionine, and the Nonidet P-40 extracts were immunoprecipitated with anti-H-2^d antiserum. The resulting two-dimensional polyacrylamide gel electrophoretic pattern is shown in Fig. 3*A*. Sixteen LMP with mobilities identical to those found in immunoprecipitates of P388D1 and WEHI-3 are also present in the normal spleen cell immunoprecipitates. This experiment thus indicates that the molecular species responsible for the adsorbing activity of spleen cell suspensions are, in fact, identical to those recognized in the macrophage cell lines.

Direct precipitation from labeled spleen cells was also used to confirm the typing by adsorption for all the strains listed in Table 1 and the intra-*H-2* recombinants, as indicated in Table 2.

Polymorphism. The two-dimensional gel patterns obtained by direct precipitation from BALB/c (H-2^d) and B10.M (H-2^f) spleen cells are compared in Fig. 3. Only one of the 16 LMP displays a shift in mobility between these two haplotypes. Gel patterns of the other positive haplotypes (*a*, *k*, *s*, and *ja*) are all identical to that obtained with B10.M (data not shown).

Tissue Distribution. Cell lines derived from a variety of tissue types were examined by direct precipitation for the presence of the LMP. A summary of the results of this analysis is shown in Table 3. All lines tested of the monocyte/macrophage lineage are strongly positive, as are normal peritoneal macrophages (unpublished observation). B, T, and null cell lines of appropriate genotype are also positive, whereas a B cell line derived from the genetically negative B10.A(5R) strain is negative. The LMP are also easily detectable on mouse L cells (fibroblasts); however, several attempts to precipitate them from the H-6 hepatoma line have failed. This result has been confirmed by adsorption experiments, which can potentially detect levels of expression too low to be seen directly by autoradiography.

Biochemical Properties. Analysis of the gel patterns in Figs. 2 and 3 indicates that the proteins described here have apparent molecular weights ranging between approximately 15,000 and approximately 30,000 and exhibit a wide range of isoelectric points (pI approximately 4 to >8). The gels also suggest that these LMP are not glycosylated; addition of neutral sugar and of sialic acid to the polypeptide chain causes an increase in apparent molecular weight and a decrease in pI, respectively, resulting in a "ladder-like" series of intermediates on two-dimensional gels. Such ladders are apparent for H-2 and Ia, but not for the LMP. Experiments using the antibiotic tunicamycin, which inhibits the addition of N-linked carbohydrate to the nascent polypeptide chain (32, 33), confirm this prediction. Whereas H-2 and Ia ladders "collapse" into single, lower molecular weight spots when cells are labeled in the presence of tunicamycin, the molecular weights of the LMP are unaffected (unpublished observations).

Subcellular Localization. Most membrane proteins are glycosylated. Thus, their lack of glycosylation suggested that the LMP may not be expressed at the cell surface. Data from a series of experiments indicates that this is indeed the case [E. Sung and P. Jones, personal communication (for a detailed description of techniques, see ref. 34)]: (*i*) The LMP are not labeled by lactoperoxidase-catalyzed iodination of cell surface proteins;

Table 2. Genetic mapping of LMP

		Haplotype of origin*									
			I								
Strain	H-2 type	*K*	*A*	*B*	*J*	*E*	*C*	*S*	*D*	Adsorption	Precipitation
B10.A(1R)	*h1*	*k*	*k*	*k*	*k*	*k*	*d*	*d*↵	*b*	+	ND
B10.HTI	*i*	*b*	*b*	*b*	*b*	*b*	*b*	*b*↵	*d*	−	ND
B10.A(5R)	*i5*	*b*	*b*	*b*↵	*k*	*k*	*d*	*d*	*d*	−	−
B10.A(4R)	*h4*	*k*	*k*↵	*b*	*b*	*b*	*b*	*b*	*b*	+	+
B10.GD	*g2*	*d*	*d*↵	*b*	*b*	*b*	*b*	*b*	*b*	+	ND
B10.AQR	*y1*	*q*	↵*k*	*k*	*k*	*k*	*d*	*d*	*d*	−	−
B10.MBR	*bq1*	*b*	↳ *k*	*k*	*k*	*k*	*k*	*k*	*q*	ND	+

ND, not done.

* The genotypes of the individual *H-2* subregions are shown. Vertical bars indicate the position of the crossover event, and arrows indicate to which side of the crossover the LMP locus maps.

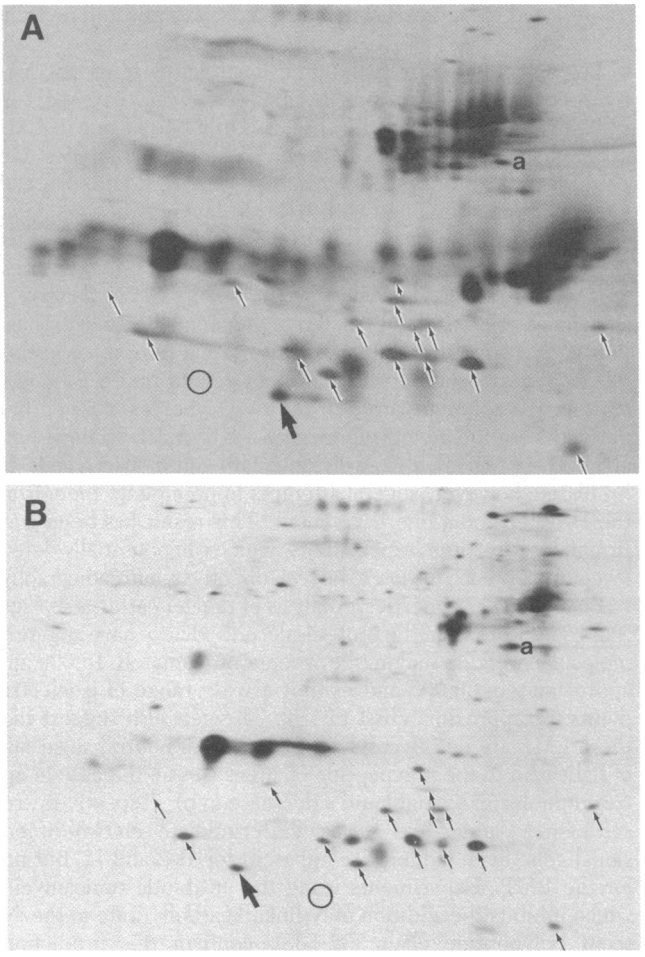

FIG. 3. Two-dimensional polyacrylamide gel electrophoretic analysis of [^{35}S]methionine-labeled spleen cell extracts precipitated with anti-H-2^d from BALB/c (*A*) and B10.M (*B*) mice. Fifteen of the 16 LMP are identical in the two panels (arrows). One of the 16 spots is unique to each strain (heavy arrow). The position of the corresponding missing spot on each gel is indicated by the open circle. Fluorographs are deliberately overexposed to visualize the weaker spots.

(*ii*) they are not present in preparations of isolated membranes washed free of cytoplasm; and (*iii*) they are not precipitated when intact labeled cells are incubated with antibody and then washed free of excess antibody prior to Nonidet P-40 solubilization and addition of fixed *S. aureus*.

DISCUSSION

We have described here the characterization of a previously undetected set of proteins. Analysis of three independently derived sets of H-2 congeneic mice demonstrated tight linkage between the genes controlling the expression of these proteins and the *H-2* complex. Thus, transfer of the *H-2^b* or *H-2^k* haplotypes onto the BALB/c background or *H-2^b* onto the C3H background results in cotransfer of the donor alleles for these proteins. Likewise, transfer of several independent or recombinant *H-2* haplotypes (*a, d, k, ja, f, s, h1, h4, g2,* and *bq1*) onto the nonexpressor B10 background results in congeneic strains that express the LMP. Thus, in the derivation of 13 of the 13 strains thus far tested, the locus controlling LMP expression has been cotransferred with the selected marker (H-2). This constitutes overwhelming evidence for tight genetic linkage between the two markers. The data shown in Table 2 for the intra-*H-2* recombinants are all consistent with the LMP locus being centromeric to the *I-A* subregion. A single recombinant, B10.MBR, suggests that this locus lies to the right (telomeric) of *H-2K*, placing it between the *K* and *I-A* subregions. The right- and left-hand boundaries would thus be defined by the *y1* and *bq1* recombinational events, respectively. However, although we consider it unlikely (because the LMP locus has not been recombinationally separated from *H-2K* in any of the other 12 strains tested), we cannot rule out the possibility of a double crossover event in the derivation of the B10.MBR strain, which may have resulted in (*H-2^k* type) genetic information from the B10.AKM parental strain being inserted centromeric to the *H-2K* region. Therefore, although we can say with some certainty that the LMP locus is centromeric to *I-A*, its assignment to the *K* to *I-A* interval must be regarded as tentative. This assignment can be examined with a four-point linkage test.

It should also be noted here that the B10.AQR, B10.MBR, and B10.A(1R) strains demonstrate that the LMP locus is genetically separable from, respectively, the *I* and *S*, *K*, and *D* regions of *H-2*. This observation effectively rules out the trivial possibility that the LMP are simply proteolytic breakdown products of any of the previously defined products of the *H-2* complex.

Although we have demonstrated that the LMP are separable from class I, II, and III antigens, we have been unable thus far

Table 3. Tissue distribution of LMP

Cell line	Strain of origin	H-2 type	Tissue type	Ref.	LMP precipitation
WEHI-3	BALB/c	*d*	Myelo-monocyte	22	+++
P388D1	DBA/2	*d*	Macrophage	23	+++
J774	BALB/c	*d*	Macrophage	24	+++
RAW264	BALB/c	*d*	Macrophage	25	+++
PU5-1.8	BALB/c	*d*	Monocyte	26	+++
BALENLM17	BALB/c	*d*	B cell	27	+
BCL1	BALB/c	*d*	B cell	28	+
T69	B10.A(5R)	*i5*	B cell	*	–
BW5147	AKR	*k*	T cell	29	+
LSTR-A	BALB/c	*d*	Null cell	30	+
LMTK$^-$	C3H	*k*	Fibroblast	31	++
H6	A/J	*a*	Hepatoma	†	–

* Unpublished observations.
† Obtained from Jackson Laboratory.

to separate them, either genetically or antigenically, from each other—all strains tested express either all or none of the 16 peptides (we have been unable to detect any products from the *b* and *q* haplotypes), and we have found no antiserum that will precipitate some but not all of these proteins. However, although their biochemical properties are similar, their lack of glycosylation and large charge separation would suggest that they are not simply related to each other by post-translational modification. Pulse–chase and *in vitro* translation experiments should help to resolve these questions.

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