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J Immunol 1979;123;182-188

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

0022-1767/79/1231-0182$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 123, No. 1, July 1979
Printed in U.S.A.

GENETIC CONTROL OF IMMUNE RESPONSE TO SPERM WHALE MYOGLOBIN IN MICE

II. T Lymphocyte Proliferative Response to the Synthetic Antigenic Sites[1]

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The genetic control of T lymphocyte proliferative response to the five synthetic antigenic sites of myoglobin, two synthetic nonantigenic control peptides, and one "nonsense" peptide was determined in independent and recombinant strains of mice. In all the strains examined, the nonantigenic control peptides and the "nonsense" peptide did not invoke a response in myoglobin-primed mice. Further, when mice were not primed with whole myoglobin, no response was obtained with any of the antigenic sites. Haplotypes *H-2*^{d}, *H-2*^{f}, and *H-2*^{s} are higher responders to sites 1 and 2, whereas haplotypes *H-2*^{d} and *H-2*^{s} are high responders to site 5. Response to site 3 may be controlled by a non-H-2-linked gene. Site 4 can stimulate *H-2*^{b} and *H-2*^{k} haplotypes that are nonresponders to the whole myoglobin. Studies with the recombinant strains suggested that Ir genes to sites 1 and 2 map in the I-A subregion and I-C subregion and were designated Ir-Mb-1,2(A) and Ir-Mb-1,2(C). Ir genes to sites 4 and 5 mapped only in the I-A subregion and were designated Ir-Mb-4(A) and Ir-Mb-5(A). These studies suggest that individual antigenic sites in a molecule are controlled by unique Ir genes.

Studies in a variety of species have revealed that the immune response to various antigens are under control of immune response (Ir)[2] genes (1–4). Immune response to thymus-dependent antigens are controlled by genes in the I region of the major histocompatibility complex (MHC) of mice (1). The specificity of Ir gene control for synthetic antigens and native proteins can be distinguished at the primary sequence level of the antigen (5–8). Ir-gene defects may be expressed on macrophages and B cells as well as T cells (9). Most of the antigens studied to date for Ir-gene control have been synthetic polymers of a few amino acids. Only recently, genetic studies were extended to include several natural protein antigens such as staphylococcal nuclease (10), myoglobin (11, 12), and cytochrome *c* (13).

Received for publication September 25, 1978.
Accepted for publication April 9, 1979.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by grants from the National Institute of Allergy and Infectious Diseases (AI 14764), National Cancer Institute (CA 24473), National Institute of Arthritis and Metabolic Diseases (AM 18920), and the Mayo Foundation.
[2] Abbreviations used in this paper: Ir, immune response; MHC, major histocompatibility complex; PPD, purified protein derivative; PETLES, peritoneal exudate T lymphocyte-enriched cells.

In our laboratories we have initiated studies on the genetic control of the immune response in mice to sperm whale myoglobin and to the antigenic sites on this protein. Sperm-whale myoglobin is made up of a single polypeptide chain (14) (153 residues, m.w. 17,816), and its entire antigenic structure has been determined (15). The native protein has five antigenic sites located in exposed conformationally sensitive continuous portions of its polypeptide chain (Fig. 1). The five antigenic sites occupy the following regions: *Site 1:* sequence 15 through 22; *Site 2*: sequence 56 through 62; *Site 3*: sequence 94 through 99; *Site 4*: sequence 113 through 119; *Site 5*: sequence 145 through 151 (15). These antigenic sites were determined by using antibodies made against whole sperm whale myoglobin in rabbits and goats. We wanted to know whether mice would recognize the same antigenic sites. If they do, the availability of congenic and recombinant strains of mice would enable us to more definitively study and map the gene(s) controlling the immune response to individual antigenic sites.

In previous studies, we found that T lymphocyte proliferative response to the intact myoglobin molecule was under H-2-linked Ir-gene control (12). We identified at least two genes mapping in *I-A* and *I-C* subregions. Our present experiments revealed that different mouse strains recognize different determinants on the molecule, and the T cell proliferative responses to sites 1, 2, 4, and 5 are controlled by genes mapping in the *I-A* subregion. Additional Ir genes for sites 1 and 2 also mapped in the *I-C* subregion. This study does not rule out the possibility that mice might recognize antigenic sites in other parts of the molecule not recognized by rabbit or goats. However, preliminary studies have shown that the antibodies to myoglobin made in three strains of mice (*H-2*^{d}, *H-2*^{s}, and *H-2*^{f}) bind to the expected antigenic sites. The total binding activity of the reacting sites accounted for almost 100% of the mouse antimyoglobin antibody (Twining *et al.*, in preparation).

MATERIALS AND METHODS

Mice. The inbred strains, the recombinants, and the F_1 hybrids used in this study were produced in our immunogenetics mouse colony at Mayo Clinic. Some of the inbred strains were purchased from Jackson Laboratories, Bar Harbor, Maine. All mice used in these experiments were between 8 and 16 weeks of age at the start of immunization.

Antigens. Myoglobin used in these studies was the major chromatographic component No. 10 (MbX) obtained by chromatography of the crystalline protein on CM-cellulose (16). MbX was homogeneous by starch gel and by polyacrylamide disc electrophoresis. The peptides synthesized and studied in the present work are shown in Table I. They comprise the five antigenic sites of myoglobin (15), two reference or control

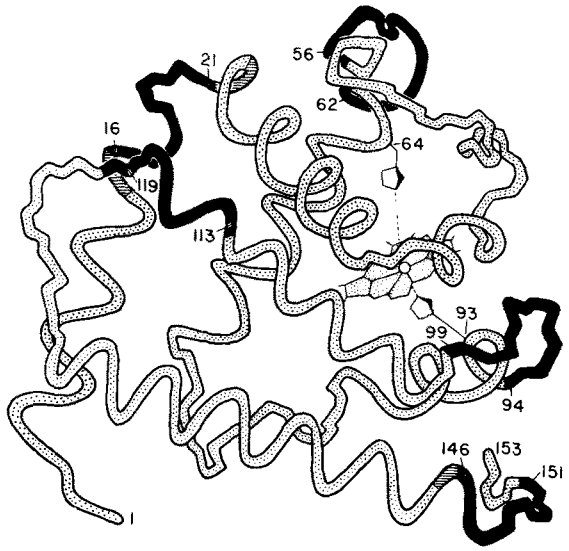

Figure 1. A schematic diagram showing the mode of folding of Mb and its antigenic structure. The solid black portions represent segments which have been shown to comprise accurately entire antigenic sites. The striped parts, each corresponding to one amino acid residue only, can be part of the antigenic site with some antisera. The dotted portions represent parts of the molecule which have been shown exhaustively to reside outside antigenic sites. (From Atassi (15); reproduced with permission from Pergamon Press).

peptides representing nonantigenic parts of the myoglobin molecule (15), and a control "nonsense" peptide whose sequence was designed so that it does not resemble any part of the myoglobin molecule, even at the dipeptide level. The synthesis and purification of the myoglobin peptides 15 through 22 (17), 56 through 62 (18), 94 through 99 (19), 113 through 120 (20), and 145 through 151 (21) have already been described in detail. The control nonantigenic peptide 1 through 6 was synthesized and purified as previously described (19). Synthesis and purification of the control nonantigenic peptide 121 through 127 and of the "nonsense" peptide were performed by similar procedures. All peptides used in this work were homogeneous in heavily loaded peptide "maps" showing purity of 99% or better. The amino acid composition of each peptide was in excellent agreement with that expected from its structure (Fig. 1). Purified protein derivative of tuberculin (PPD) was purchased from Connaught Medical Research Labs., Willowdale, Ontario, Canada.

Immunization. Dose response studies were first initiated to determine the optimum amount of myoglobin for priming. A dose of 50 μg gave an optimum proliferative response (12). Eight mice of each strain were immunized with 50 μg of myoglobin mixed 1:1 in complete Freund's adjuvant containing 1 mg/ml of *Microbacterium tuberculosis*, strain H37Ra (Difco Labs., Detroit, Mich.). The mixture was injected in the two hind foot/pads, and the animals were sacrificed 3 weeks later.

Cultures. The preparation of peritoneal exudate T-lymphocyte-enriched cells (PETLES) and their *in vitro* culture have been described in detail by Schwartz *et al.* (22). In brief, thioglycolate-induced peritoneal exudate cells were passed through nylon-wool columns. The cells were cultured at 2.5×10^5 per well in U-type microtiter plates in 0.2 ml of a modified Eagles-Hanks' media (23) containing 10% heat-inactivated fetal calf serum for 72 hr in the presence or absence of antigen at the final concentration of 40 μg/ml. The stimulation was assessed by measuring the incorporation of a 2 μCi pulse of tritiated thymidine (specific activity 1.9 Ci/mM, New England Nuclear, Boston Mass.) 16 hr before termination of the cultures. Each PETLES experiment was run in triplicate culture wells and the PETLES assay on each strain was tested three to six times. The data are expressed as mean counts per minute (cpm) plus or minus standard deviation (S.D.).

RESULTS

Dose-response studies. Studies with whole myoglobin showed that 50 μg of the protein with Freund's complete adjuvant was the optimum dose for the T lymphocyte proliferative response (12). The stimulation indices obtained with this optimum dose were, however, only a fraction of that obtained with PPD. The dose of each site that would optimize the proliferative response of sensitized PETLES in the culture wells was also determined. Strains BALB/c ($H\text{-}2^d$), B10.K ($H\text{-}2^k$), B10.S ($H\text{-}2^s$), and B10.M ($H\text{-}2^f$) were challenged with each site at the dosage level of 10, 20, 40, 80, and 160 μg/ml. The stimulation was measured at 48, 72, and 96 hr. The results on BALB/c and B10.K are shown in Figures 2 and 3. All our experiments represent a 72-h culture period since the 96-hr cultures gave high backgrounds. Varying the whole myoglobin dose 1/200th to ½ on a molar basis relative to that of the peptide did not significantly change the cpm.

Response of independent haplotypes. The T lymphocyte proliferative response to individual antigenic sites was tested in mouse strains of all independent haplotypes. The mice were primed *in vivo* with the whole myoglobin and then challenged *in vitro* with each of the synthetic peptides of the antigenic sites 1 through 5, the two control sites, and the "nonsense" peptide. PPD was included in each experiment, and response in each strain varied between 15,000 to 130,000 cpm. The data are not included in the table to conserve space. Each strain was tested approximately six times in the PETLES assay. Since occasionally we obtained either no stimulation or extremely high stimulation, the lowest and highest counts were discarded. Experiments involving very low PPD responses were repeated. The data shown represent the median cpm and stimulation

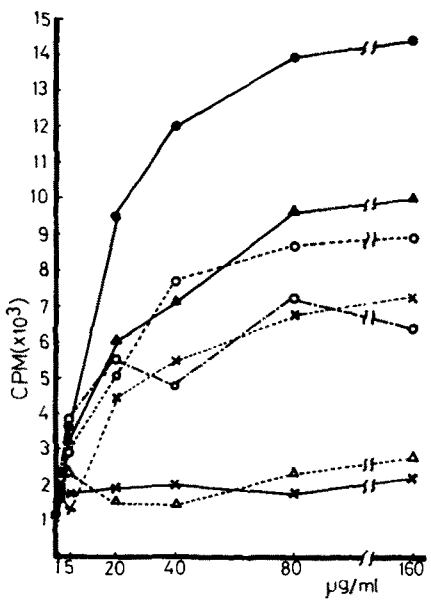

Figure 2. Dose response curve of T-lymphocyte proliferative response to myoglobin antigenic sites. BALB/c by PETLES sensitized with whole myoglobin were cocultured with each concentration of antigenic sites; site 1 (O–·–·–O); site 2 (O-----O); site 3 (▲——▲), site 4 (△-----△), site 5 (×-----×) and nonsense peptide (×——×) as well as whole molecule (●——●). Each point represents the mean of three replicates.

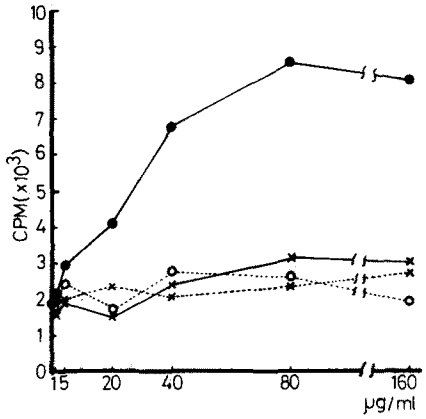

Figure 3. Dose response curve of T-lymphocyte proliferative response to myoglobin antigenic sites. B10.K PETLES sensitized with whole myoglobin were cocultured with whole myoglobin (○-----○), site 1 (×——×), site 2 (×-----×), and site 4 (●——●). Each point represents the mean of three replicates.

TABLE I

Sequence of synthetic peptides

Synthetic Antigenic Sites	
Site 1	15 22 Ala-Lys-Val-Glu-Ala-Asp-Val-Ala
Site 2	56 62 Lys-Ala-Ser-Glu-Asp-Leu-Lys
Site 3	94 100 Ala-Thr-Lys-His-Lys-Ile-Pro
Site 4	113 120 His-Val-Leu-His-Ser-Arg-His-Pro
Site 5	145 151 Lys-Tyr-Lys-Glu-Leu-Gly-Tyr
Synthetic Control Non-Antigenic Regions	
Control 1	1 6 Val-Leu-Ser-Glu-Gly-Glu
Control 2	121 127 Gly-Asn-Phe-Gly-Ala-Asp-Ala
Synthetic "Nonsense" Peptide	
	Glu-Ser-Ser-Gly-Thr-Gly-Ile

index of the middle four values and the range of stimulation indices. Stimulation indices were calculated by equating the response to "nonsense" peptide as 1.00. A strain was classified as a responder if the cpm was twice as much as seen with the nonsense peptide.

As shown in Tables I, II, and III, of the *H-2*^{b} haplotype strains tested (B10, B6, and A.BY), B10 gave a weak response to the whole myoglobin whereas B6 and A.BY did not respond at all. Among the sites, site 4 stimulated, whereas sites 1, 2, and 5 failed to stimulate, in all three *H-2*^{b} haplotypes. Even though the SI for site 4 in *H-2*^{b} haplotypes was not very high, this was highly repeatable and confirmed in several other strains (B6-K1, B6-K2, B6(TL+), and AKR-*H-2*^{b}). Among the *H-2*^{d} haplotypes, B10.D2, BALB/c, and DBA/2 responded substantially to the whole myoglobin as well as sites 1, 2, and 5. None of the *H-2*^{d} mice tested responded to site 4. BALB/c gave a substantial response to site 3 whereas DBA/2 gave only a weak response. The *H-2*^{f} haplotype mice of strain B10.M responded to the whole molecule as well as sites 1, 2, and 3, but failed to respond to sites 4 and 5. The *H-2*^{k} haplotype strains, B10.BR and B10.K,

TABLE II

T-lymphocyte proliferative response in independent haplotypes to myoglobin antigenic sites [Cpm (mean ± S.D.)][a]

Strain	B10	B6	A.BY	B10.D2	Balb/cBY	DBA/2	B10.M	B10.WB	B10.BR	B10.K	B10.P	B10.G	B10.RIII	B10.S	A.SW
Haplotype	b	b	b	d	d	d	f	j	k	k	p	q	r	s	s
Ag(−)	745 ± 82	988 ± 35	743 ± 62	1,262 ± 138	1,407 ± 218	2,031 ± 605	1,604 ± 187	1,834 ± 260	925 ± 241	3,988 ± 133	2,945 ± 119	4,344 ± 850	6,010 ± 516	1,995 ± 278	625 ± 865
Whole Mb	2,034 ± 261	1,542 ± 126	933 ± 74	7,862 ± 416	16,011 ± 2,938	6,723 ± 1,273	8,142 ± 278	2,432 ± 655	938 ± 236	5,160 ± 961	5,049 ± 140	6,527 ± 541	6,608 ± 603	8,655 ± 1,460	7,304 ± 618
Site 1	1,244 ± 267	1,300 ± 270	873 ± 137	10,263 ± 1,347	6,810 ± 409	12,804 ± 518	4,895 ± 507	2,171 ± 369	1,011 ± 197	3,694 ± 566	3,851 ± 44	5,798 ± 408	4,518 ± 552	5,361 ± 193	5,200 ± 374
Site 2	1,053 ± 23	1,096 ± 456	866 ± 30	18,047 ± 536	7,144 ± 1,062	10,532 ± 1,207	4,064 ± 714	1,894 ± 357	912 ± 60	6,054 ± 390	3,912 ± 258	6,944 ± 771	4,951 ± 146	7,403 ± 726	4,948 ± 558
Site 3	966 ± 130	2,135 ± 149	809 ± 76	949 ± 209	5,836 ± 322	4,062 ± 336	5,089 ± 271	2,544 ± 116	787 ± 213	6,255 ± 403	4,106 ± 137	3,385 ± 1,307	4,520 ± 362	5,870 ± 426	6,250 ± 1,024
Site 4	2,743 ± 65	2,899 ± 93	1,833 ± 451	1,519 ± 85	2,309 ± 596	3,144 ± 277	1,683 ± 273	2,065 ± 254	2,203 ± 289	9,746 ± 417	3,788 ± 286	2,887 ± 539	4,577 ± 407	3,796 ± 288	4,129 ± 479
Site 5	865 ± 49	811 ± 108	1,072 ± 295	7,962 ± 788	5,819 ± 779	14,225 ± 3,965	1,578 ± 545	1,867 ± 306	929 ± 325	6,235 ± 28	2,970 ± 303	5,346 ± 279	4,168 ± 894	6,052 ± 573	1,266 ± 257
Control 1	812 ± 136	989 ± 66	790 ± 174	1,190 ± 444	1,486 ± 253	3,564 ± 524	2,264 ± 65	2,458 ± 96	790 ± 137	4,059 ± 631	2,564 ± 462	5,165 ± 946	3,779 ± 162	2,511 ± 193	1,148 ± 536
Control 2		1,246 ± 152	834 ± 131	818 ± 326	1,618 ± 216	3,051 ± 632	2,153 ± 135	2,144 ± 350	835 ± 365	6,800 ± 523	2,514 ± 255	6,074 ± 210	3,785 ± 650	2,714 ± 66	1,092 ± 98
Nonsense	1,196 ± 148	1,154 ± 263	785 ± 219	1,834 ± 16	1,413 ± 328	2,998 ± 738	3,049 ± 645	1,676 ± 145	1,092 ± 674	4,102 ± 396	2,904 ± 279	5,641 ± 293	4,914 ± 580	1,845 ± 255	918 ± 125

[a] About ten mice of each strain were injected with 50 μg of Mb with complete Freund's adjuvant in footpads. After 19 days, 2.5×10^5 PETLES were cultured with Mb and its antigenic sites at the final concentration of 40 μg/ml. After 4 days, ^{3}H-thymidine-incorporated cells were harvested. PPD (40 μg/ml) response of each strain was between 15,000 ~ 130,000 cpm.

like the *H-2*^{b} haplotype strains, failed to respond to the whole myoglobin and to sites 1, 2, 3, and 5, but did give a significant response to site 4. This was again confirmed in other *H-2*^{k} strains (C3H, CBA, C58, B6-*H-2*^{k}, AKR, and BRVR). Strains B10.WB (*H-2*^{j}), B10.P (*H-2*^{p}), B10.G (*H-2*^{q}), and B10.RIII (*H-2*^{r}) failed to respond to the whole myoglobin as well as to any of the sites. The *H-2*^{s} haplotype strains B10.S and ASW responded to the whole myoglobin as well as sites 1, 2, and 5. Strain ASW, in addition, responded to site 3. The results obtained on the independent haplotypes indicate that: 1) haplotypes *H-2*^{d}, *H-2*^{f}, and *H-2*^{s} are high responders to site 1 and 2. In addition, haplotypes *H-2*^{d} and *H-2*^{s} are also high responders to site 5; 2) response to site 3 may be controlled by a non-H-2 linked gene; and 3) site 4 can stimulate *H-2*^{b} and *H-2*^{k} haplotypes, which are nonresponders to the whole myoglobin.

In all the strains examined, the nonantigenic peptides did not stimulate a T lymphocyte proliferative response in myoglobin-primed mice. Unprimed mice did not give T lymphocyte proliferative response when challenged with any of the antigenic sites.

Response in H-2 recombinants. In order to map the genes controlling immune responses to individual antigenic sites of sperm whale myoglobin, we tested selected H-2 recombinants involving high and low responder strains (Table IV). Strain

TABLE III

Stimulation indices of independent haplotypes to myoglobin antigenic sites[a]

Strain Haplotype	B10 b	B6 b	A.BY b	B10.D2 d	BALB/cBY d	DBA/2 d	B10.M f	B10.WB j	B10.BR k	B10.K k	B10.P p	B10.G q	B10.RIII r	B10.S s	A.SW s
Nonsense	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Whole Mb	1.8 (1.5–2.7)	0.9 (0.5–1.3)	1.5 (1.0–2.0)	4.3 (2.4–6.3)	11.6 (11.4–12.0)	2.6 (2.2–2.9)	5.0 (2.6–9.7)	1.7 (1.5–2.3)	1.0 (0.8–1.2)	1.3 (1.1–1.7)	1.6 (1.3–1.7)	1.2 (1.2–1.3)	1.5 (1.4–1.8)	3.4 (2.1–4.4)	4.8 (3.9–5.8)
Site 1	1.1 (0.9–1.5)	1.0 (0.7–1.1)	1.1 (1.1–1.3)	4.5 (3.0–5.6)	4.6 (4.1–4.9)	3.4 (2.5–4.3)	2.3 (1.6–3.1)	1.6 (1.0–2.6)	1.0 (0.8–1.1)	1.0 (0.8–1.2)	1.4 (1.2–1.7)	1.0 (0.9–1.1)	1.2 (0.9–1.6)	4.3 (0.7–9.5)	3.3 (2.6–4.1)
Site 2	0.9 (0.5–1.1)	0.9 (0.6–1.3)	0.9 (0.8–1.1)	5.4 (1.8–9.8)	4.3 (1.2–5.8)	3.2 (2.9–3.5)	2.4 (1.2–2.7)	1.3 (1.0–1.9)	1.1 (0.9–1.5)	1.4 (1.2–2.0)	1.3 (0.9–1.5)	1.3 (1.3–1.4)	1.1 (0.8–1.4)	3.9 (1.5–6.5)	3.3 (2.6–3.9)
Site 3	0.8 (0.7–1.0)	1.6 (0.9–2.0)	1.3 (1.0–1.5)	4.2 (2.3–8.0)	3.8 (3.2–4.2)	2.3 (1.4–3.3)	2.0 (1.6–2.7)	1.7 (1.0–2.6)	0.9 (0.7–1.3)	1.3 (0.6–2.1)	1.2 (0.6–1.5)	0.8 (0.7–1.5)	1.1 (0.9–1.2)	1.8 (1.6–2.6)	3.8 (0.7–3.7)
Site 4	2.2 (1.7–3.1)	2.0 (1.5–2.5)	2.0 (1.7–2.3)	0.8 (0.7–0.8)	1.4 (1.0–1.6)	1.1 (1.1–1.2)	1.1 (0.6–1.5)	1.4 (0.8–2.3)	2.0 (1.7–2.1)	2.4 (2.0–3.2)	1.3 (1.0–1.4)	0.8 (0.6–1.0)	1.1 (0.9–1.2)	1.4 (0.7–1.9)	1.7 (1.2–2.3)
Site 5	0.7 (0.6–0.7)	0.8 (0.7–1.1)	1.3 (1.1–1.4)	2.5 (1.4–4.3)	5.2 (4.1–7.2)	4.6 (4.5–4.7)	0.8 (0.5–1.0)	1.1 (0.6–1.8)	1.0 (0.9–1.1)	1.4 (1.1–2.1)	1.0 (0.8–1.2)	1.2 (1.1–1.3)	1.0 (0.9–1.2)	2.1 (1.0–3.1)	2.5 (1.0–3.5)
Control 1	0.7 (0.7–0.7)	1.0 (0.6–1.6)	0.9 (0.7–1.7)	0.7 (0.6–0.8)	1.0 (0.7–1.2)	1.1 (1.1–1.2)	0.8 (0.7–0.9)	0.8 (0.6–1.5)	0.8 (0.7–1.0)	1.1 (0.9–1.3)	0.9 (0.6–1.4)	1.2 (1.0–1.4)	1.2 (1.0–1.4)	1.0 (0.7–1.3)	0.9 (0.9–0.9)
Control 2	1.0 (0.9–1.1)	1.0 (0.7–1.2)	1.1 (1.1–1.2)	0.8 (0.4–1.2)	1.0 (0.9–1.1)	1.1 (1.0–1.2)	0.7 (0.7–0.7)	1.3 (0.6–2.0)	0.8 (0.6–0.8)	1.3 (0.7–2.3)	0.9 (0.7–1.2)	1.2 (1.1–1.3)	0.8 (0.6–1.0)	1.4 (1.0–1.4)	0.9 (0.7–1.0)

[a] Each strain was tested at least three times. The median stimulation index and the range for each strain is shown. The stimulation indices were calculated by making the cpm for nonsense peptides as 1.00. SI = $\frac{\text{test antigen cpm}}{\text{nonsense peptide cpm}}$. SI with PPD varied between 15 and 100.

TABLE IV

Stimulation indices of selected recombinants and F_1 to myoglobin antigenic sites[a]

Strain H-2 haplotype	A.TL t1	A.TH t2	B10.HTT t3	D2.GD g2	B10.A a	B10.A (2R) h2	B10.A (5R) i5	B10.S (8R) as	B10.A (4R) h4	B10.S (9R) t4	C3H.OL o′	ATFR·5 ap5	(B10.S × B10.BR)F_1 s × k	(B6 × B10.K)F_1 b × k
Nonsense	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Whole Mb	1.1 (1.0–1.2)	3.0 (2.1–3.7)	7.1 (3.5–11)	3.0 (2.3–4.2)	3.4 (1.4–4.7)	2.8 (2.7–2.9)	3.9 (2.2–5.7)	1.3 (1.0–1.9)	1.8 (1.5–1.8)	8.0 (3.2–11.2)	3.1 (2.6–3.8)	3.2 (2.6–4.7)	2.5 (2.0–2.7)	1.1 (1.0–1.2)
Site 1	1.1 (0.9–1.3)	2.8 (1.6–3.0)	3.6 (1.0–7.3)	2.2 (1.6–3.0)	2.1 (1.5–2.4)	2.5 (1.8–3.6)	5.0 (3.3–8.9)	1.3 (0.8–1.7)	1.5 (1.3–1.6)	4.0 (1.6–6.1)	2.0 (1.7–2.4)	3.1 (1.7–4.5)	3.1 (2.0–4.5)	1.2 (1.0–1.5)
Site 2	1.0 (1.0–1.0)	2.5 (1.0–3.0)	3.2 (1.5–6.3)	3.3 (1.7–5.4)	2.7 (1.2–5.3)	2.4 (1.7–3.5)	1.9 (1.2–2.4)	1.4 (0.6–2.3)	1.4 (1.3–1.5)	4.9 (3.4–7.9)	2.4 (1.2–2.9)	3.2 (1.9–6.4)	2.7 (1.8–4.5)	1.2 (1.0–1.4)
Site 3	1.1 (1.0–1.2)	1.1 (0.8–1.4)	1.1 (1.0–1.2)	1.1 (1.0–1.3)	2.2 (1.2–4.0)	1.7 (1.1–2.7)	2.8 (1.7–3.9)	1.2 (0.7–1.6)	1.3 (1.3–1.4)	1.6 (0.8–2.3)	2.4 (2.0–3.0)	2.9 (2.0–4.3)	2.4 (2.1–3.2)	1.0 (0.9–1.0)
Site 4	2.9 (1.7–3.0)	1.1 (0.9–1.4)	1.0 (0.9–1.0)	0.9 (0.8–1.1)	2.4 (1.5–3.3)	2.5 (1.7–3.5)	2.1 (1.2–2.7)	3.0 (1.1–5.3)	2.2 (2.0–2.4)	0.6 (0.6–0.7)	1.6 (0.9–2.2)	1.3 (1.1–1.8)	2.3 (1.9–3.0)	2.0 (1.9–2.2)
Site 5	0.8 (0.6–0.9)	2.2 (1.9–2.4)	2.9 (1.6–4.6)	2.6 (1.0–4.0)	1.0 (0.7–1.4)	0.9 (0.8–0.9)	1.2 (1.0–2.9)	1.2 (0.8–1.6)	1.7 (1.3–2.0)	3.0 (0.7–5.6)	2.6 (2.1–3.3)	1.1 (0.9–1.3)	3.0 (2.2–3.6)	1.4 (0.8–1.7)
Control 1	1.0 (0.8–1.1)	1.0 (1.0–1.0)	1.3 (0.6–1.4)	1.1 (0.9–1.2)	1.1 (0.5–1.7)	1.1 (0.7–1.5)	1.1 (1.0–1.2)	0.8 (0.7–1.0)	1.3 (1.2–1.3)	1.2 (0.6–2.2)	1.6 (0.9–2.7)	1.4 (1.1–2.0)	1.3 (1.0–1.6)	1.0 (0.8–1.4)
Control 2	1.1 (0.9–1.2)	1.4 (1.3–1.4)	1.0 (0.9–1.2)	1.3 (0.9–2.0)	1.1 (0.8–1.6)	1.2 (0.9–1.6)	1.4 (1.1–1.7)	1.1 (0.7–1.3)	1.4 (1.1–1.6)	1.3 (0.9–1.6)	1.6 (1.2–2.6)	1.3 (0.9–1.5)	1.1 (1.0–1.2)	1.1 (1.0–1.1)

[a] Each strain was tested at least three times. The median stimulation index and the range for each strain is shown. The stimulation indices were calculated by making the cpm for nonsense peptides as 1.00. SI = $\frac{\text{test antigen cpm}}{\text{nonsense peptide cpm}}$. SI with PPD varied between 15 and 100.

A.TL responded only to site 4 as did the $H\text{-}2^k$ haplotype, whereas A.TH strain mice responded to sites 1, 2, and 5 as the $H\text{-}2^s$ haplotype. Since A.TL and A.TH differ only between *H-2K* and *H-2D* the Ir genes for myoglobin sites 1, 2, 4, and 5 map in *I*, *S*, or *G* regions. Strain B10.HTT, which carries the high responder $H\text{-}2^s$ alleles in *I-A*, *I-B*, and *I-J*, responded to sites 1, 2, and 5. Since B10.S(9R) ($I\text{-}J^k$) also gave similar results, mapping of Ir genes for sites 1, 2, and 5 was further narrowed down to the *I-A* and/or *I-B* subregions. The gene controlling responses to site 1, 2, and 5 specifically mapped to the *I-A* subregion since recombinant D2.GD responded to these sites similarly to $H\text{-}2^d$ haplotype. D2.GD carries the $H\text{-}2^b$ haplotype genes to the right of *I-A*, and mice of this haplotype do not respond to these sites. Since D2.GD failed to respond to site 4 to which $H\text{-}2^b$ responds; the Ir gene to site 4 can also be mapped to the *I-A* subregion. Recombinants B10.A, B10.A(2R), and B10.A(5R) responded to sites 1 and 2, whereas B10.A(4R) and B10.S(8R) failed to respond. Since $H\text{-}2^k$ haplotype also does not respond to sites 1 and 2, a second Ir gene to sites 1 and 2 can be mapped between the *I-E* subregion and the *H-2D* subregion. The recombinants C3H.OL and C3H.OH, which express the *S* and *G* regions from low and high responders, respectively, gave similar responses to sites 1 and 2; the influence of these regions must be minimal. On the basis of these recombinants, a second Ir gene to myoglobin sites 1 and 2 can be tentatively mapped to the *I-C* subregion. The results with the H-2 congenic strains suggested that the Ir genes to myoglobin site 3 might be associated with non-H-2 linked gene. Among the recombinants, B10.A and B10.A(5R) responded to site 3, whereas B10.A(2R) failed to respond. This suggests that response to site 3 may be influenced by some genes mapping in the TL region.

Several F_1 crosses were made involving combination of responders and nonresponders. Crosses between responders and nonresponders always responded to the whole myoglobin as well as to the sites suggesting dominant Ir-gene control. In matings involving two nonresponder strains, we did not see any evidence for complementation of Ir genes for response to the antigenic sites.

Results of experiments testing several other recombinant strains confirmed the mapping of Ir genes to the myoglobin antigenic sites. B10.HTG and B10.BDR (I^d) gave results similar to the $H\text{-}2^d$ haplotype mice. B10.A(18R), B10.A(21R), B10.BAR(5), B10.D2(R106), B10.D2(R107), A-BTR-1, B10.F(14R), and HTI (I^b) gave results similar to the $H\text{-}2^b$ haplotype mice, in that they responded only to site 4. B10.M(17R) and B10.AQR ($I^{k/d}$) gave results similar to the $H\text{-}2^a$ haplotype. A.TFR-1 (I^k), A.AL, A.AKR, B10.AM, and B10.AKM responded to site 4 similar to A.TL. B10.M(11R) and A.TFR-5 (I^f) gave results similar to the $H\text{-}2^f$ haplotype. B10.T(6R) (I^q) gave results similar to $H\text{-}2^q$ haplotype, and B10.S(7R) and B10.S(24R) (I^s) gave results similar to $H\text{-}2^s$ strains. All the mutants that were typed gave results that were characteristic of the parental strains. For example, B6-$H\text{-}2^{bgl}$, B6-$H\text{-}2^{bh}$, and B10.M505 responded to site 4 similar to the B6 ($H\text{-}2^b$). M504 gave results similar to B10.D2 ($H\text{-}2^d$), and M523 responded to site 4 similar to $H\text{-}2^k$ haplotypes.

DISCUSSION

The present studies clearly showed that T lymphocyte proliferative response to sperm whale myoglobin and four of the five antigenic sites are under control of H-2-linked *Ir* genes. In Table V, we have used the key strains to summarize the mapping of the Ir genes to myoglobin antigenic sites. Figure 4 depicts the mapping of these Ir genes. So far, we have been unable to separate the Ir genes to sites 1 and 2, which have always been expressed together. The Ir genes to the myoglobin sites will be designated as follows: 1) *Ir-Mb-1,2(A)* for the gene responding to sites 1 and 2, mapping in the *I-A* subregion; 2) *Ir-Mb-1,2(C)* for the gene responding to sites 1 and 2 mapping in the *I-C* subregion; 3) *Ir-Mb-3* for the gene(s) responding to site 3, which is non-H-2-linked; 4) *Ir-Mb-4(A)* for the gene responding to site 4, mapping in the *I-A* subregion; and 5) *Ir-Mb-5(A)* for the gene responding to site 5, mapping in the *I-A* subregion. One interesting point is the response of recombinant B10.S(8R), which carries the *I-C* subregion from $H\text{-}2^s$ haplotype, a high responder, failed to respond to sites 1, 2, and 5. This suggests that $H\text{-}2^d$ haplotype expresses both *Ir-Mb-1,2(A)* and *Ir-Mb-1,2(C)*,whereas $H\text{-}2^s$ haplotype expresses only the *Ir-Mb-1,2(A)*. The *I-E/I-C* gene products have not been detected in $H\text{-}2^s$ haplotype.

The expression of either *Ir-Mb-1,2(A)* or *Ir-Mb-1,2(C)* is sufficient for response to sites 1 and 2. This suggests that individual Ir genes can respond to distinct antigenic sites on the molecule. Gene complementation could occur by additive response of two Ir genes responding to different sites on the molecule. In the parent strain, response generated by one of the Ir genes may not reach the threshold value to be classified as a responder. Another area of interest is the response of $H\text{-}2^k$ and $H\text{-}2^b$ haplotypes to site 4, although they failed to respond to the whole myoglobin molecule. There are two possible explanations: 1) Dosage effect: when the primed lymphocytes of $H\text{-}2^k$ and $H\text{-}2^b$ haplotypes are challenged *in vitro* with the site 4, they are exposed at optimum dose to approximately 18 times more antigen compared to challenge with the whole molecule. If this were true, increasing the dosage of myoglobin 10- to 20-fold during challenge should make $H\text{-}2^b$ and $H\text{-}2^k$ high responders. It is significant that these strains remained low responders

TABLE V

Summary of response to myoglobin antigenic sites in selected recombinants

Strain	K	A	B	J	E	C	S	G	D	Response
B10	b	b	b	b	b	b	b	b	b	4
B10.D2	d	d	d	d	d	d	d	d	d	1, 2, 3, 5
D2.GD	d	d	b	b	b	b	b	b	b	1, 2, 5
C3H.OL	d	d	d	d	d	d	k	k	k	1, 2, 3, 5
B10.K	k	k	k	k	k	k	k	k	k	4
A.TL	s	k	k	k	k	k	k	k	d	4
B10.A	k	k	k	k	k	d	d	d	d	1, 2, 3, 4
B10.A(2R)	k	k	k	k	k	d	d	?	b	1, 2, 4
B10.A(5R)	b	b	b	k	k	d	d	d	d	1, 2, 3, 4
B10.S	s	s	s	s	s	s	s	s	s	1, 2, 5
B10.S(9R)	s	s	?	k	k	d	d	d	d	1, 2, 5
B10.HTT	s	s	s	s	k	k	k	k	d	1, 2, 5
B10.S(8R)	k	k	?	?	s	s	s	s	s	4

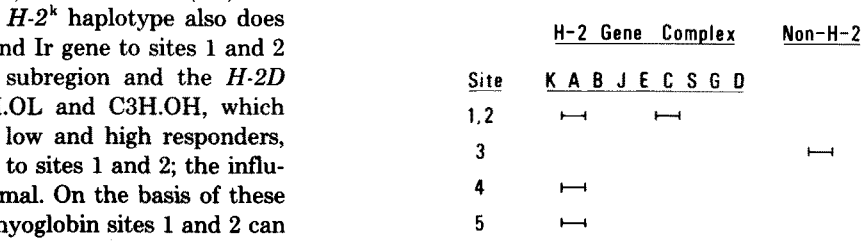

Figure 4. Ir gene mapping for individual antigenic sites of sperm whale myoglobin.

when the myoglobin dose was varied from 1/200th to ½ relative to that of the peptide. Thus the dosage effect may be ruled out. 2) Suppressive site: there might be a site on the myoglobin molecule that gives suppressive signals in $H\text{-}2^k$ and $H\text{-}2^b$ haplotypes. When the mice are exposed to site 4 only, in the absence of this putative suppressive signal, they are able to proliferate. This hypothesis can also be experimentally verified by challenging with couplets of site 4 and the other sites. Studies with the sites in the priming process might also reveal some interesting information.

The entire antigenic structure of sperm whale myoglobin was determined originally by Atassi on the basis of the antibody response in rabbits and goats. An initial question was whether the mice would recognize the same antigenic sites. The T cell proliferation studies indicate that the mice recognize the same sites. We also considered the possibility that the mice might recognize additional sites on the molecule. However, preliminary studies have also shown that the antibodies to myoglobin made in three strains of mice bind to the expected antigenic sites. The total binding activity of the reacting sites accounted for almost 100% of the mouse antimyoglobin antibody (Twining *et al.*, in preparation).

Studies with synthetic amino acid polymers have suggested that Ir-gene control at the T cell level was carrier dependent. However, the present studies suggest that this need not be necessarily true and that the recognition could be at the antigenic site level. Presumably Ir-gene control can be mediated through several avenues. Even though studies with amino acid polymers have yielded very important information on the immune response gene phenomena, there are also certain pitfalls. For example, synthetic amino acid polymers prepared from *N*-carboxy-amino acid anhydrides would be expected to differ from one preparation to another, and also a given preparation does not represent a discrete molecular species. These chemical differences would lead to changes in the antigenic properties of the molecule and the mechanism by which it is recognized. Studies with protein antigens should generate new information on the Ir-gene mechanisms.

The T cell proliferation and the antibody response to the whole molecule gave similar results. Studies in several laboratories (24–26) have suggested that T cells recognize different determinants from those recognized by B cells. The T cell responses are believed to be specific for amino acid sequence on fragments of native protein antigens, whereas antibodies are directed against the tertiary structure of the antigenic sites on the native antigen. It is therefore significant to point out that the control synthetic peptides, representing nonantigenic parts of the molecule, consistently failed to stimulate a T cell proliferative response in all the numerous strains examined. It would be interesting now to see whether the antibody response to the free synthetic sites (which would be expected to be completely unfolded in solution) in appropriate strains would give similar results as shown in this paper for the T cell proliferation. Can antibodies be produced to the synthetic antigenic sites in the high responder mice by immunization with each of these sites or is the tertiary structure required for antibody production? Of course, it should not be ruled out that binding receptors could induce the free peptide into the appropriate conformation as do antibodies (15). Further, at the antigenic determinant level, are the T cell repertoire and B cell repertoire similar or different? These are questions that can be answered by doing antibody-binding assays and the T cell proliferative assays in the same strains of mice by using the synthetic antigenic sites.

Unanue and Cerottini (27) have established that macrophages phagocytize antigens and put out antigen fragments that bind to their surface membranes. Recently, Benacerraf (28) has proposed that specific Ir-gene function dictates the ability of the Ia molecule on macrophages and B cells to interact specifically with unique amino acid sequences on the antigen concerned. This would generate the determinants specific for T cell recognition and would suggest that the Ia molecules possess binding sites that are complementary to an antigen segment of a specific amino acid sequence. Applying this idea to our studies, it would mean that the Ia molecules generated by genes coded in the *I-A* subregion of $H\text{-}2^d$ haplotype can bind to sites 1, 2, and 5, whereas the Ia molecule from $H\text{-}2^k$ haplotype have complementary surface structure for binding only site 4. This hypothesis is being experimentally tested in our laboratories.

Acknowledgments. The authors thank Suresh Savarirayan for superb mouse husbandry, and Sharon Ames and Connie Batt for skillful preparation of the manuscript.

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