Brief Definitive Report

DIFFERENCES IN MATERNAL LINEAGES OF NEW ZEALAND BLACK MICE DEFINED BY RESTRICTION ENDONUCLEASE ANALYSIS OF MITOCHONDRIAL DNA AND BY EXPRESSION OF MATERNALLY TRANSMITTED ANTIGEN*

By MARILYN M. HUSTON, ROGER SMITH III, DAVID P. HUSTON‡, AND ROBERT R. RICH

From the Departments of Medicine and Microbiology & Immunology and the Howard Hughes Medical Institute, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030

New Zealand Black (NZB)[1] mice are an autoimmune, hyperresponsive strain important in the study of autoimmunity and immunoregulation (1, 2). Recently, attempts to characterize target antigens for cytolytic T lymphocyte (CTL) responses from NZB anti-*H-2*-identical mixed leukocyte cultures have led to the identification of a new alloantigen, called maternally transmitted antigen (Mta) (3–5). Mta is a cell surface antigen found on lymphocytes from most mouse strains (3); it is recognized by *H-2*-nonrestricted CTL from the few Mta^- strains, NZB/BlN, NZO, or NMRI (3). Interestingly, two substrains of NZB mice, NZB/BlPt and NZB/Füll do express Mta (3, 4). The expression of Mta is determined by maternal phenotype (Mta^+ or Mta^-) and cannot be modified by foster mothering, embryo transfer, or transfer of bone marrow cells to lethally irradiated mice (3, 6). Fischer Lindahl et al. (7) have shown that the Mta phenotype of a maternal ancestor is maintained through eleven generations of backcrossing. Because the inheritance of Mta is maternally determined and not consistent with chromosomal inheritance, it is likely that Mta expression is cytoplasmically transmitted, perhaps through a cell-associated virus or cytoplasmic organelle such as mitochondria (3, 6). The only known cell surface phenotypic difference between NZB substrains is the expression of Mta. We sought a cytoplasmic marker to distinguish between the possibilities that these differences in Mta expression reflect (*a*) separate maternal lineages, or (*b*) spontaneous mutation. Such a distinction is important to understanding the possible extra-nuclear control of cell surface alloantigen expression.

Because mitochondria are cytoplasmically and thus maternally inherited (8), restriction endonuclease analysis of mitochondrial DNA (mtDNA) can be used to characterize maternal lineage. It has recently been reported that all of the "old inbred" strains studied (BALB/cJ, C57BL/6J, C58/J, DBA/2N, SWR/J, AU/SsJ, C3H, PL/J, and AKR/Cum), unlike wild mice or mice of other unrelated inbred strains, have an identical mtDNA restriction endonuclease fragment pattern. These

* Supported in part by grants 1-K08-AI-00519-01 and AI-13810 from the National Institutes of Health.

‡ Recipient of Clinical Investigator Award 1-K08-AI-00519-01 from the National Institutes of Health.

[1] *Abbreviations used in this paper:* CAS, supernatants of Con A-stimulated splenocytes; CTL, cytolytic T lymphocyte; Mta, maternally transmitted antigen; mtDNA, mitochondrial DNA; NZB/BlN, New Zealand Black/Bielschowsky origin, mice from the NIH colony; NZB/BlNJ are NZB/Bl mice originally from the NIH colony, now obtained from The Jackson Laboratory; NZB/BlPt are NZB/Bl mice from Michael Potter's separate colony also at the NIH.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/83/06/2154/06 $1.00
Volume 157 June 1983 2154–2159

identical patterns indicate either derivation of these strains from a single maternal parent or subsequent contamination of the original breeder lines (9). We have used Mta-specific CTL clones and restriction endonuclease digestion followed by electrophoretic separation of mtDNA to analyze two substrains of NZB mice that differ with respect to Mta expression. In contrast to the "old inbred" strains, the Mta^- strain NZB/BlN showed a unique mtDNA restriction enzyme pattern, indicating a maternal lineage distinct from that of the other strains characterized. Conversely, NZB/BlPt, which are Mta^+, presented a pattern indistinguishable from that of the "old inbred" type. These findings are indicative of genetic contamination of the NZB/BlPt substrain and are consistent with changes in their Mta phenotype.

Materials and Methods

Mice and Cell Lines. NZB/BlN mice were obtained from the NIH breeding colony. NZB/BlPt mice were obtained from M. Potter, NIH. NMRI mice were obtained from the Naval Medical Research Institute (NMRI) Bethesda, MD. NZW/LacJ, DBA/2J, B10.D2/nSnJ, and B10.BR/SgSnJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME. BALB/cCrgl, (NZB/BlNJ × B10.BR)F_1 and (B10.BR × NZB/BlNJ)F_1[2] mice were bred in the Baylor College of Medicine animal facilities.

mtDNA Preparation. mtDNA was isolated from partially purified mitochondria by the method of Portnoy et al. (10). Briefly, livers, spleens, and kidneys were collected from three to five mice. The tissues were minced on ice in mannitol sucrose buffer, 0.21 M mannitol, 0.7 M sucrose, 0.005 M Tris pH 7.3, 0.005 M EDTA, and the cells were disrupted with three to four strokes of a Potter-Elvehjem homogenizer with Teflon pestle. Nuclei were pelleted at 1,000 *g* for 15 min at 4°C. Mitochondria were pelleted from the supernate by centrifugation twice at 8,700 *g* at 4°C for 25 min. The pellet was resuspended in its residual volume and layered over a discontinuous gradient of 1.2 ml 1.5 M sucrose and 3 ml 1 M sucrose in 0.1 M Tris pH 7.2, 0.01 M KCl, 0.005 M EDTA, and centrifuged for 1 h at 37,000 rpm in a SW50.1 rotor at 4°C. The mitochondrial band was collected, resuspended in mannitol sucrose, and pelleted at 10,800 *g* at 4°C for 20 min. The pellet of purified mitochondria was resuspended in the residual volume and the mtDNA isolated by alkaline lysis (10). The DNA preparation was phenol chloroform-extracted twice and ethanol-precipitated before restriction endonuclease analysis. A typical mtDNA preparation from four to five adult mice was enough for six to eight digests.

Restriction Endonuclease Analysis. mtDNA was digested overnight at 37°C with the restriction endonucleases Hinf 1 or Mbo 1 using the digestion mixture suggested by the supplier, New England Biolabs, Beverly, MA. The size standard, phage ϕX174 digested with Hae 111, was supplied predigested. The samples were run on vertical 30 mm × 15 mm × 0.75 mm slab gels of 5% polyacrylamide in the Tris borate buffer system of Maniatis et al. (11). The gels were stained by the silver staining methods developed by Merrill et al. (12) and Beidler et al. (13).

Generation of Long-term CTL Lines. A detailed description of these techniques will be provided elsewhere.[3] Briefly, NZB/BlNJ spleen cells were primed in vivo and then cultured for 6 d with irradiated BALB/c or B10.D2 spleen cells. Effector cells harvested from these cultures were enriched for viable cells with a density gradient (Lympholyte-M, Accurate Chemical & Scientific Corp., Westbury, NY) and cloned by limiting dilution on irradiated cells from the immunizing strain in medium supplemented with supernatants of 24-h cultures of Concanavalin A-stimulated BALB/c splenocytes (CAS). The lines were derived from cultures initially seeded with 10 viable responder cells from the 6-d mixed leukocyte culture. Generally, fewer than 20–25% of such cultures scored positive for lysis of target cells after 3–4 wk of culture. The cell lines were expanded and propagated in culture by addition of irradiated BALB/c or B10.D2 splenocytes and fresh CAS.

CTL Assay. Assay of CTL was performed as previously described (5). Target cells were 60–72-h Concanavalin A blasts labeled with $Na^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL) and added to serial dilutions of the CTL lines. After a 4-h incubation at 37°C, the plates were

[2] By convention the maternal strain is listed first.

[3] Jenkins, R. N., and R. R. Rich. Characterization of Qa-1 determinants and alleles by cloned cytolytic T lymphocytes. Manuscript in preparation.

centrifuged at 200 *g* for 5 min, and harvested with the Titertek Supernatant Collection System (Flow Laboratories, Inc., McLean, VA). Data are expressed as % net ^{51}Cr release = 100 × (experimental release-spontaneous release)/(maximum release-spontaneous release), where the spontaneous release is the activity released from targets in the absence of CTL, and maximum release is the activity released from targets by three cycles of freezing and thawing.

Results

Phenotyping with Cloned Mta-Specific CTL. Fig. 1 depicts the results of an Mta-phenotyping experiment using the NZB/BlNJ CTL line 35C10. The Mta specificity of 35C10 is demonstrated by differential lysis of reciprocal F_1 targets. (NZB/BlNJ × B10.BR)F_1 mice, like their Mta^- NZB/BlNJ mother, are Mta^-, regardless of the sex of the progeny. The reciprocal (B10.BR × NZB/BlNJ)F_1 mice, derived from Mta^+ B10.BR mothers, are all Mta^+. Lysis of the Mta^+ F_1 target cells, in the absence of lysis of the Mta^- F_1 targets, demonstrates the specificity of this clone for Mta. These cloned Mta-specific CTL were then used to Mta phenotype spleen cells from the NZB substrains, as well as NZW, BALB/c, and NMRI. The failure to lyse NZB/BlNJ and NZB/BlN targets confirms these substrains as Mta^-. In contrast, lysis of the NZB/BlPt targets demonstrates that this substrain is Mta^+. The CTL line 35C10 also lyses targets from NZW, BALB/c and NMRI mice, phenotyping these strains as Mta^+. Lysis of the NZW ($H\text{-}2^z$) targets demonstrates that the CTL line 35C10 recognizes Mta in an *H-2*-nonrestricted manner. Similar results were obtained using six other independent Mta-specific CTL lines.

Restriction Endonuclease Analyses of mtDNA. Fig. 2 shows the banding patterns of Hinf 1 restriction endonuclease cleavage of mtDNA from NZB, NZW, BALB/c, and NMRI mice. The banding patterns for the cleaved mtDNA from NMRI, BALB/c, NZW, and the NZB/BlPt strains are identical. By contrast, the Hinf 1-generated pattern of NZB/BlN mtDNA is different. There are five major and at least three minor differences in the molecular weight of the Hinf 1-generated fragments from NZB/BlN mtDNA versus mtDNA from the other strains. Four additional low molecular weight Hinf 1-generated fragments are predicted from the known DNA sequence of mtDNA (14), but are not visualized on these gels. Using the endonuclease, Mbo 1, at least four molecular weight differences were observed between mtDNA from NZB/BlPt and NZB/BlN mice (data not shown). The Mbo 1 cleavage patterns of NZB/BlPt and BALB/c were identical to each other and to the pattern published by Ferris et al. (9) for all of the "old inbred" strains of mice.

Discussion

We used cloned Mta-specific CTL and restriction endonuclease digestion of mtDNA to analyze phenotypic differences between NZB substrains. The cloned Mta-specific CTL recognized alloantigens on NZB/BlPt cells but not on NZB/BlN. NZB/BlN mice had a different mtDNA restriction endonuclease pattern, using Hinf 1 and Mbo 1, from the common pattern shared by the "old inbred" strains. In contrast, NZB/BlPt mice had the same pattern as the "old inbred" strains. NZB/BlPt mice have a substantially modified autoimmune disease, including a delayed onset of Coombs' positivity, reduced titers of anti-DNA antibodies, and prolonged survival.[4] In addition, these mice can be readily tolerized, in contrast to NZB/BlN.[5] However, Mta is the only cell surface antigen for which expression is known to differ between

[4] A. D. Steinberg, personal communication.
[5] D. Lewis, personal communication.

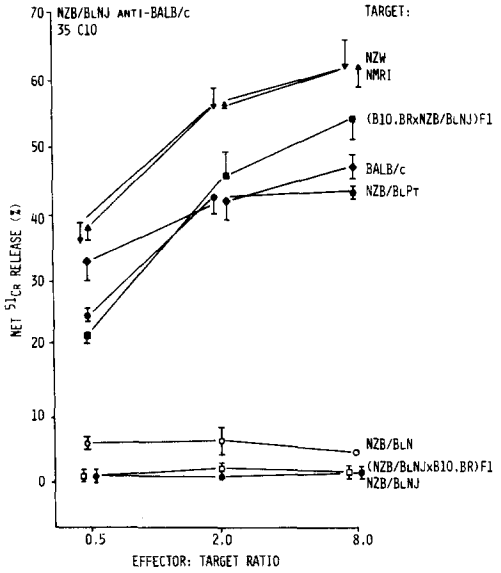

FIG. 1. NZB/BlNJ anti-BALB/c CTL line 35C10 was assayed for lysis of target cells from the indicated strains. Spontaneous release values were 8–20% of the maximum release for each target with 20,000 target cells per well. The vertical bars represent one standard error of the % net ^{51}Cr release.

NZB/BlN and NZB/BlPt mice. It was possible that the phenotypic differences in autoimmune disease and Mta expression in NZB/BlPt mice were due to the spontaneous mutation of the relevant genes or to genetic contamination with another mouse strain. Our results demonstrate that the NZB/BlPt substrain has been contaminated with one of the "old inbred" strains. This probably explains their altered autoimmune status and is consistent with changes in their Mta phenotype. Thus the NZB/BlPt substrain should probably be regarded as a separate recombinant inbred strain between NZB and an unknown gene donor in the maternal lineage.

Although Fischer Lindahl et al. reported NMRI mice from European stocks to be Mta^- (6), the NMRI mice we tested from the Naval Medical Research Institute were Mta^+ by cloned CTL assay and shared the same mtDNA phenotype with the common "old inbred" strains. That NMRI mice have the "old inbred" type of mtDNA is not surprising since SWR is one of the "old inbred" strains and both SWR and NMRI descended from Swiss mice obtained from Lausanne and passaged by C. Lynch (15). NZW mice, which we found to exhibit the "old inbred" type of mtDNA and which are Mta^+, are not reported to be related to NZO or NZB mice, the latter of which strains were derived from a common stock (15). It is not possible to determine whether NZW share a common maternal lineage with the "old inbred" strains, or whether they have been subsequently contaminated. The demonstration of the apparent acquisition of the "old inbred" type of mtDNA by strains not known to be genetically related to the early inbred strains of mice (15), probably serves as another example of the difficulty in maintaining the genetic purity of inbred mouse strains (9, 16).

The mtDNA phenotype represented by NZB/BlN mice is not necessarily unique but merely contrasts with the lack of restriction site polymorphism of mtDNA in the other strains tested. Using Mbo 1 digestion, we found that NZB/BlN mtDNA appears to be similar to mtDNA found in several different wild mice (9). Likewise, the "old

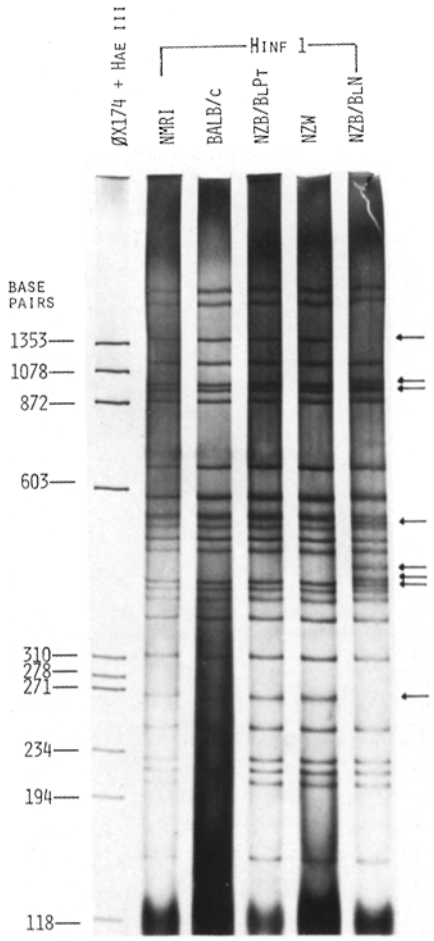

FIG. 2. Restriction endonuclease digests of mtDNA from different strains of mice using Hinf 1. The ϕX174 Hae III fragment sizes have been determined from sequenced DNA. Arrows indicate differences in Hinf 1 recognition sites between NZB/BlN and the several other strains of mice examined (NMRI, BALB/c, NZB/BlPt, and NZW), the latter of which all demonstrated identical restriction patterns.

inbred" type of mtDNA is not always found in mice with an Mta^+ phenotype. SF/Cam mice are reported to be Mta^+ (7), but have an mtDNA restriction endonuclease pattern using Mbo 1 that is different from both the "old inbred" strains (9) and from NZB/BlN. Positive correlation between the mtDNA phenotype of NZB/BlN and failure to express the Mta antigen would require the testing of more Mta^- strains and wild mice. However, such experiments would provide only indirect evidence for the involvement of a mitochondrial gene in Mta expression. Mitochondrial transfer experiments in progress should more directly assess this relationship as causal or coincidental.

Summary

Two substrains of New Zealand Black (NZB) mice have been compared with respect to expression of a maternally transmitted cell surface antigen, Mta, defined by cloned cytolytic T cells, and for restriction enzyme polymorphisms of mitochondrial DNA (mtDNA). These independent assays of maternal cytoplasmic inheritance provide strong evidence for genetic contamination of the NZB/BlPt substrain

(NZB/Bl mice from Michael Potter's separate colony at the National Institutes of Health), in which the typical NZB immunologic abnormalities are at least partially ameliorated. The decisive data are the restriction enzyme maps of mtDNA for NZB/BlPt, which were identical with those of the common "old inbred" strains and quite different from those of NZB/BlN (NZB/Bl mice from the breeding facility at the National Institutes of Health). It is probable that the contamination of the NZB/BlPt substrain is related to phenotypic changes in their autoimmune state. More interestingly, the data are consistent with, although they do not prove, involvement of the mitochondrial genome in expression of a cell surface molecule.

The authors with to thank Dr. Richard Hull and Dr. Mark Entman for sharing thoughts and equipment and Dr. K. Fischer Lindahl for generously sharing prepublication data. The secretarial assistance of Martha Roy and Sue Floyd was appreciated.

Received for publication 17 February 1983.

References

1. Izui, S., P. J. McConahey, and F. J. Dixon. 1978. Increased spontaneous polyclonal activation of B lymphocytes in mice with spontaneous autoimmune disease. *J. Immunol.* **121**:2213.
2. Huston, D. P., and A. D. Steinberg. 1980. NZB cytotoxic lymphocyte responses: kinetic analysis. *J. Exp. Med.* **152**:748.
3. Fischer Lindahl, K., M. Bocchieri, and R. Riblet. 1980. Maternally transmitted target antigen for unrestricted killing by NZB T lymphocytes. *J. Exp. Med.* **152**:1583.
4. Fischer Lindahl, K., and J. Langhorne. 1981. Medial histocompatibility antigens. *Scand. J. Immunol.* **14**:643.
5. Smith, R., III, D. P. Huston, and R. R. Rich. 1982. Primary cell-mediated lympholysis response to a maternally transmitted antigen. *J. Exp. Med.* **156**:1866.
6. Fischer Lindahl, K., and K. Burki. 1982. Mta, a maternally inherited cell surface antigen of the mouse, is transmitted in the egg. *Proc. Natl. Acad. Sci. USA.* **79**:5362.
7. Fischer Lindahl, K., and B. Hausman. 1983. Cytoplasmic inheritance of a cell surface antigen in the mouse. *Genetics.* **103**:483.
8. Dawid, I. B., and A. W. Blackler. 1972. Maternal and cytoplasmic inheritance of mitochondrial DNA in Xenopus. *Dev. Biol.* **29**:152.
9. Ferris, S. D., R. D. Sage, and A. C. Wilson. 1982. Evidence from mtDNA sequences that common laboratory strains of inbred mice are descended from a single female. *Nature (Lond.).* **295**:163.
10. Portnoy, D. A., S. L. Moseley, and S. Falkow. 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia entercolitica* pathogenesis. *Infect. Immun.* **31**:775.
11. Maniatis, T., A. Jeffrey, and H. Van deSande. 1975. Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. *Biochemistry.* **14**:3787.
12. Merrill, C. R., M. L. Dunau, and D. Goldman. 1981. A rapid sensitive silver stain for polypeptides in polyacrylamide gels. *Anal. Biochem.* **110**:201.
13. Beidler, J. L., P. R. Hilliard, and R. L. Rill. 1982. Ultrasensitive staining of nucleic acids with silver. *Anal. Biochem.* **126**:374.
14. Bibb, M. J., R. A. Van Etten, L. T. Wright, M. W. Walberg, and D. A. Clayton. 1981. Sequence and gene organization of mouse mitochondrial DNA. *Cell.* **26**:167.
15. Staats, J. 1981. Inbred and segregatory inbred strains. *In* The Mouse in Biomedical Research. Vol. 1. H. L. Foster, J. D. Small, and J. G. Fox, editors. Academic Press, New York. pp. 177–213.
16. Kahan, B., R. Auerbach, B. J. Alter, and F. H. Bach. 1982. Histocompatibility and isoenzyme differences in commercially supplied "BALB/c" mice. *Science (Wash. DC).* **217**:379.