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MOUSE MAMMARY TUMOR VIRUS (MMTV), A RETROVIRUS THAT EXPLOITS THE IMMUNE SYSTEM

GENETICS OF SUSCEPTIBILITY TO MMTV INFECTION

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Summary: All animals, including humans, show differential susceptibility to infection with viruses. Study of the genetics of susceptibility or resistance to specific pathogens is most easily studied in inbred mice. We have been using mouse mammary tumor virus (MMTV), a retrovirus that causes mammary tumors in mice, to study virus/host interactions. These studies have focused on understanding the mechanisms that determine genetic susceptibility to MMTV-induced mammary tumors, the regulation of virus gene expression *in vivo* and how the virus is transmitted between different cell types. We have found that some endogenous MMTVs are only expressed in lymphoid tissue and that a single base pair change in the long terminal repeat of MMTV determines whether the virus is expressed in mammary gland. This expression in lymphoid cells is necessary for the infectious cycle of MMTV, and both T and B cells express and shed MMTV. Infected lymphocytes are required not only for the initial introduction of MMTV to the mammary gland, but also for virus spread at later times. Without this virus spread, mammary tumorigenesis is dramatically reduced. Mammary tumor incidence is also affected by the genetic background of the mouse and at least one gene that affects infection of both lymphocytes and mammary cells has not yet been identified. The results obtained from these studies will greatly increase our understanding of the genetic mechanisms that viruses use to infect their hosts and how genetic resistance to such viruses in the hosts occurs.

Key words: MMTV, mammary tumor, superantigen

Mouse mammary tumor virus (MMTV) is a type B retrovirus that causes mammary carcinomas in mice[1]. The use of MMTV as a model system has been critical to the study of many different aspects of cancer, gene regulation and developmental biology. MMTV was the first transmissible agent shown to cause cancer in mammals[2, 3] and since its discovery has been widely used as a model for breast cancer. MMTV was also the first mammalian "gene" shown to encode DNA sequences that caused increased transcription in response to glucocorticoid hormones[4]. Thus, the original proof that mammalian transcription factors interact with specific DNA sequences came about from studies of how glucocorticoid receptors bound to and induced MMTV expression. Tumorigenesis by MMTV is mediated by integration near cellular oncogenes; a number of different genes located at different integration sites (called *int* genes) have been implicated in this process[5-9]. These novel oncogenes, some of which are developmentally important, were identified solely by their associa-

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tion with MMTV-induced mammary tumors. At least one of the unique oncogenes, *int*-2, a member of the fibroblast growth factor family, is activated in some human mammary cancer[10, 11]. Thus, there have been major scientific gains in many fields made by studying this virus.

MMTV has two routes of infection in mice; susceptible strains acquire the virus through milk-borne infection and can be freed of this route of infection by foster-nursing on non-viremic mothers, while other strains inherit an endogenous copy of the provirus that is activated in the mammary gland *in situ* and cannot be freed of the virus by foster-nursing[12, 13]. Although endogenous MMTV proviruses are present in the germ line of all inbred mice and there are multiple proviral sequences found at different chromosomal locations in different mouse strains, the majority do not produce virus because of mutations.

Although the ultimate target for MMTV is the mammary gland, cells of the immune system play a role in milk-borne virus infection[14, 15], as described in more detail elsewhere in this issue (Piazzon et al., p 21). MMTV is able to infect lymphocytes because it encodes a cell surface superantigen (Sag) protein in its 3' LTR[16, 17]. Sags are presented by the major histocompatibility (MHC) class II proteins of antigen presenting cells (APCs), such as B cells, to $CD4^+$ T cells bearing specific Vβ chains of the T cell receptor (TCR). This presentation of Sag causes proliferation of specific Vβ-bearing T cells when it is recognized as foreign[16] and deletion of such T cells when it is recognized as self[17]. Different proviruses cause the deletion or stimulation of different classes of Vβ-bearing T cells, because they encode Sag proteins with different C-terminal amino acid sequences (termed the hypervariable region); this region of the Sag protein contacts the TCR Vβ molecule[18-21].

Virus spread within mice that acquire MMTV through milk-borne infection requires Sag activity. The Sag causes the stimulation of cognate T cells and as a result, bystander B cells are also stimulated to divide, setting up a reservoir of infection-competent cells. The major evidence for this pathway comes from studies done with mice lacking Sag-reactive T cells[14] or B cell-deficient mice[22]; both are resistant to milk-borne transmission of MMTV.

Mouse strains vary in their susceptibility to different MMTV isolates in both tumor incidence and latency[12]. In some cases, susceptibility or resistance is due to whether or not they have endogenous MMTVs that delete Sag cognate T cells that also respond to exogenous Sags. The major histocompatibility locus (MHC) class II genes also affect susceptibility to MMTV; mice that lack the class II I-E gene, such as C57BL, are relatively virus-resistant because of inefficient presentation of most MMTV Sags[23]. There are other as of yet unidentified loci that also result in resistance to MMTV infection and mammary tumorigenesis (see below).

Both B and T lymphocytes express and shed MMTV

To provide protection against exogenous MMTV infection, endogenous MMTVs should be expressed in APCs such as B cells, so that efficient deletion of cognate T cells is achieved. However, expression of infectious endogenous MMTVs in the mammary gland leads to mammary tumorigenesis, as in the case of GR mice[24]. Thus, retention of an endogenous *Mtv* locus that is expressed in the lymphoid compartment but not in the mammary gland would be of selective advantage.

We have studied the tissue-specific expression of different MMTVs using various inbred mouse strains and RNase protection probes that target the Sag hypervariable region. As can be seen in Table 1 (taken from[25-27]), the transcriptionally active *Mtv* loci can be divided into two groups based on their tissue-specific pattern of expression. Group 1 loci (*Mtv*-1, *Mtv*-3, *Mtv*-6, *Mtv*-43) and all exogenous viruses (MMTV (C3H), BALB14 and MMTV (SW)) were expressed in both mammary gland and lymphoid tissues. In contrast, transcription from the Group 2 loci (*Mtv*-7 and *Mtv*-9) was detected only in lymphoid tissues.

We sought to determine why the different MMTVs did not show the same pattern of tissue-specific expression. We compared the LTR sequences of the different endogenous proviruses and found a region, hereafter termed the MGR, in which a single base pair change might characterize them as lymphotropic. As seen in Table 1, there is a single base pair change at position 520

TABLE 1.– *The MGRs of Different MMTVs*

Virus	Sequence*	Expression
MMTV (C3H)	C T C A A T T G A A	mammary gland/lymphoid
Mtv-1, -3, -6	C T C A G T T G A A	mammary gland/lymphoid
Mtv-43	C T C A G T C A A A	mammary gland/lymphoid
MMTV (SW)	C T C A G T C A A A	mammary gland/lymphoid
Mtv-7, -9	C G C A G T C A A A	lymphoid
BALB14	C T C A G T T G A A	mammary gland/lymphoid
REC 1[b]	C T C A G T C A A A	mammary gland/lymphoid
REC 2[c]	C T C A G T T G A A	mammary gland/lymphoid

* Sequences are between nucleotides 519 and 528, according to the numbering system of Brandt-Carlson et al., 1993. Double underlined bases denote changes from the MMTV(C3H) sequence.
[b] Recombinant between *Mtv*-7 and BALB14 with breakpoint within the MGR site.
[c] Recombinant between *Mtv*-7 and BALB14 with breakpoint 3' of the MGR site.

(relative to the 5' end of the LTR) that is characteristic of MMTVs that are expressed only in lymphoid cells. We believe that this base pair change prevents expression of *Mtv*-7 and -9 in mammary cells.

We have several pieces of evidence that show that this region does affect expression of MMTV in mammary tissue. We recently found that recombination between a newly identified exogenous MMTV, BALB14, and the *Mtv*-7 endogenous provirus found in several mouse strains, created a highly infectious and tumorigenic virus that has the *sag* gene from *Mtv*-7 ([27]; see article by Piazzon et al. p 21). Thus, although *Mtv*-7 is normally only transcribed in lymphoid tissue, the recombinant Mtv-7/BALB14 virus that retains the *Mtv*-7 *sag*, is a milk-transmitted virus. By sequence analysis of the recombinant viruses, we found that all the novel, milk-borne viruses acquired at minimum the single base pair change in the MGR from the BALB14 exogenous virus (see REC 1 and Rec 2, Table 1). This result shows that indeed this region is critical to the mammary gland expression of MMTV.

We also made transgenic mice that had the *Mtv*-7 LTR (lymphotropic), a hybrid LTR containing the MGR from *Mtv*-7 and the promoter region from MMTV(C3H) (LTR1) or a hybrid LTR containing the MGR from MMTV(C3H) and the promoter region from *Mtv*-7 linked to CAT as a reporter gene (LTR2) (Fig. 1). We previously showed that the MMTV(C3H) LTR directs expression of linked transgenes to mammary gland and lymphoid tissue[28-30]. The absolute level of transgene expression varied between the different strains due to copy number and position effects; however, the relative expression levels seen in the different tissues of the same strain were determined by the transcriptional regulatory sequences present in the transgene. We found that Mtv-7CAT mice expressed predominantly in lymphoid tissue, in contrast to MMTV(C3H)CAT transgenic mice (Fig. 1). Similarly, LTR1 transgenic mice also had higher relative levels of CAT activity in lymphoid tissue, while LTR2 mice had highest expression in mammary tissue and lower levels in lymphoid tissue (Fig. 1).

Thus, transcription of endogenous MMTVs in lymphocytes is important for the deletion of Sag-cognate T cells and for the life cycle of the exogenous form of MMTV. We showed recently that MMTV infected both B and T tissue culture cells *in vitro* and primary cells *in vivo* after milk-borne transmission of the virus[31]. The infected tissue culture cells processed viral proteins and both these and primary B and T cells shed virus when cultured *in vitro*. Moreover, the infected B and T tissue culture cells transmitted virus to uninfected mammary gland cells *in vitro*. These results indicate that both infected T and B cells are potential carriers of MMTV *in vivo*. How virus arrives at its ultimate destination, the mammary gland, is

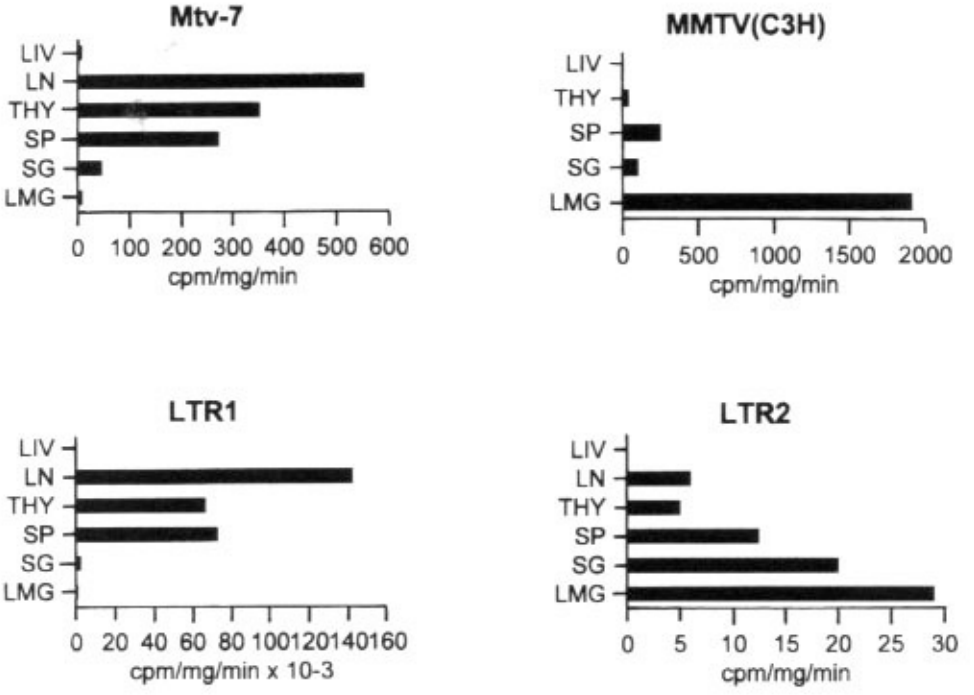

Fig. 1.– Enzymatic activity in various tissues of mice transgenic for the MMTV(C3H), Mtv-7, LTR1 or LTR2-CAT constructs. The *Mtv*-7 LTR (Mtv-7) or two different hybrid LTRs LTR1 (sequences upstream of base pair 632 from *Mtv*-7 and sequences downstream of this site from the MMTV(C3H)LTRs) and LTR2 (sequences upstream of base pair 632 from MMTV(C3H) and sequences downstream of this site from the *Mtv*-7 LTRs) were linked upstream from the CAT gene. The mice were sacrificed and CAT assays were performed with extracts prepared from various tissues as previously described[30]. The results from the MMTV(C3H)-CAT transgenic mouse were previously reported[30].
Abbreviations: LIV, liver; LN, lymph node; THY, thymus; SP, spleen; SG salivary gland; LMG, lactating mammary gland.

not clear. Preliminary studies in which either B or T cells from MMTV(C3H)-infected C3H/HeN mice were adoptively transferred into severe combined immunodeficiency (SCID) mice indicate that either subset could transmit virus to the mammary gland (not shown).

Infected lymphocytes are required for virus spread within the mamary gland

During the final part of its infectious cycle, MMTV is transcribed, translated, undergoes amplification by replication and reintegrates at novel sites in the genome of mammary gland cells. Once MMTV infects mammary gland cells, virus amplification within this tissue is required to maximize virion production and to induce mammary tumors. Mammary tumorigenesis takes place after the insertion of proviral DNA near cellular proto-oncogenes and activation of their transcription. Because retroviral integration is not site-specific, the more virions produced, the more likely it is that proviral DNA will integrate near such a proto-oncogene. MMTV transcription is induced by lactogenic hormones, including progesterone and glucocorticoids and virus production increases dramatically during pregnancy[4]. As a result, the mammary glands of virgin mice are less infected with MMTV and they have much lower mammary tumor incidence than do multiparous females[32].

We decided to investigate whether spread of virus within the mammary gland was dependent solely on hormonal induction of MMTV transcription in mammary cells or whether lymphoid cells also played a role. Although mice naturally acquire virus through milk, MMTV can also be introduced by injection into the mammary gland of pubescent mice. To determine whether MMTV injection into the mammary gland resulted in infection of

lymphoid cells, we performed virus-specific polymerase chain reaction (PCR) analysis on DNA isolated from spleen and Peyer's patches of mice infected in this manner. We found that both tissues from the infected mice acquired new proviral copies of MMTV, indicating that lymphoid cells were infected after injection into the mammary gland[33].

The first cells infected during milk-borne transmission are B cells[22]. To determine whether infected B cells were also required for MMTV spread within the mammary gland, we injected virus into mice that lacked such cells due to targeted mutagenesis of their Igμ chain gene [strain IGH6/BL6].

This gene disruption was available only in C57BL/6 mice, which are H-2^b and thus are less susceptible to MMTV (C3H) infection (see above). IGH6/BL6 mice were crossed with C3H/HeN mice, which are H-2^k, a MHC haplotype that presents the MMTV(C3H) Sag. F_1 (IgM^+/H-2^{k+}) and F_2 generation female mice (IgM^+/H-2^{k+}, IgM^+/H-2^{k-}, IgM^-/H-2^{k+}, IgM^-/H-2^{k-}) derived from these crosses received mammary gland injections of MMTV at 3-4 weeks of age. To determine whether the various mice were infected, the mice were bred and RNA isolated from their lactating mammary glands, milk and spleens at their third pregnancy was used for RNase protection assays. The IgM^+/H-2^{k+} but not the IgM^+/H-2^{k-}, IgM^-/H-2^{k-} or IgM^-/H-2^{k+} mice contained MMTV(C3H) proviruses in their splenocytes and mammary glands and shedded virus into milk (Fig. 2A).

Thus, in the absence of B cells, there was no virus spread within the immune system of mammary gland. One consequence of B cell infection by MMTV is that these cells could function as Sag-presenting APCs and activate cognate T cells. This activation would lead to amplification of virus within the lymphoid compartment. That mice of the wrong MHC haplotype failed to be efficiently infected by mammary gland injection of

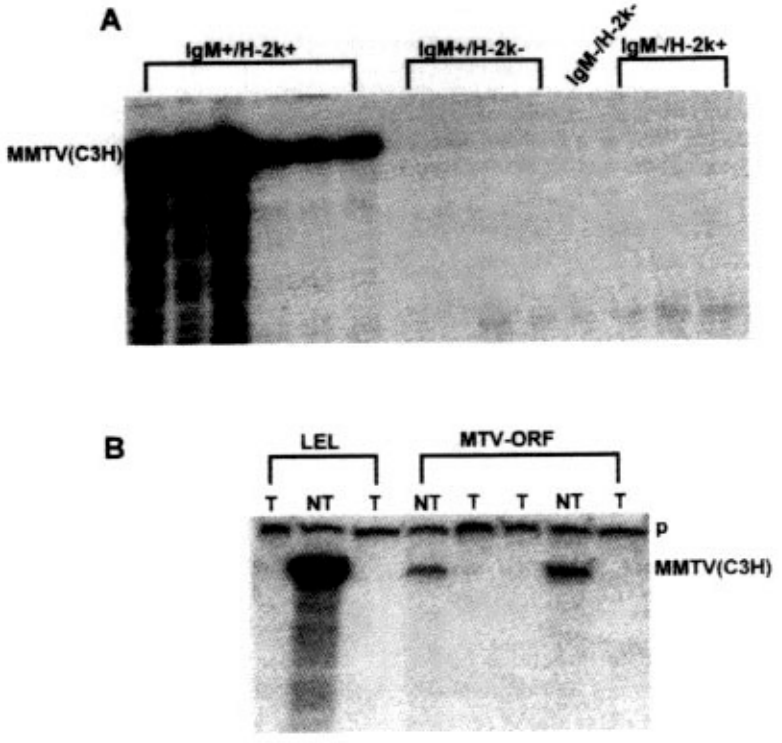

Fig. 2.– Mice lacking B cells (A) or Sag-cognate T cells (B) show no virus spread in the mammary gland. (A) Igμ knockout mice of the indicated genotypes received mammary gland injections of MMTV(C3H) at 3 to 4 weeks of age. The mice were bred and RNA isolated from milk after their third pregnancy was subjected to RNase protection analysis specific for MMTV(C3H) virus, as previously described[14]. (B) LEL or MTV-ORF transgenic mice (T) and their nontransgenic littermates (NT) were injected as in (A) and RNA isolated from their milk after the fourth pregnancy was subjected to RNase protection analysis.

MMTV indicated that Sag presentation might be a requisite step in this infection. To test directly whether Sag activity and infected lymphoid cells were required for virus spread within the mammary gland, exogenous MMTV(C3H) was injected into the mammary glands of transgenic mice that both express the MMTV(C3H) *sag* as an endogenous gene. These mice, termed MTV-ORF and LEL, lack Vβ14+T cells and thus are resistant to milk-borne MMTV (C3H) infection[14, 34].

We determined whether the mammary glands of the injected transgenic mice were MMTV-infected by isolating RNA from their milk and subjecting it to RNase T_1 protection analysis specific for MMTV(C3H) transcripts. To ensure that there was ample time for virus spread, all of the mice were analyzed after their fourth pregnancy; we have previously shown using this assay that milk-borne infection of mammary gland tissue increases with parity[14]. RNA isolated from the milk of the LEL transgenic mice injected with the high virus dose contained little or no detectable MMTV(C3H)-specific RNA, in contrast to the MMTV-injected nontransgenic mice, and no viral RNA was detected in either the LEL or MTV-ORF mice that received the lower amounts of virus (Fig. 2B).

The lack of viral amplification and consequent lower viral load in the transgenic mice had a dramatic effect on MMTV-induced mammary tumorigenesis. Because MMTV integration next to cellular oncogenes is a stochastic event, the higher the viral load, the more rapidly mice will develop mammary tumors. Both the transgenic and nontransgenic mice were continuously bred and monitored for mammary gland tumor incidence. The multiparous nontransgenic mice had a 100% mammary gland tumor incidence by the age of 260 days. At that age, none of the transgenic mice developed mammary tumors and only two out of seven transgenic mice developed tumors by 300 days. These two mice may have had sufficient infection of the mammary gland cells by direct injection, so that Sag function was not required.

These results demonstrated that infected lymphoid cells play a critical role in infection of the mammary gland. One possibility is that virus spread between mammary gland cells cannot be achieved in the absence of infected lymphoid cells. This may be because cell-cell contact between lymphocytes and mammary cells is the most efficient way to deliver virus. The architecture of the mammary gland may also affect how virus spreads. MMTV preferentially buds from the apical surface of the epithelial cells lining the alveolar lumen[35] so that the virus is released into milk during lactation. Thus, for MMTV to cause a systemic infection of the mammary gland tissue, it would have to be produced by an infected cell that directed its expression towards subepithelial tissues. B or T cells could fulfill this requirement, since both produce MMTV[31], persist within the tissue and can be activated by the cytokines produced by Sag-activated T cells[36]. Moreover, infected lymphocytes can circulate within the tissue, thereby coming into contact with mammary cells at multiple locations. These results imply that multiparous females may have higher virus loads in their mammary gland than virgins because of increased rounds of mammary cell division (i.e. the generation of more infection-competent cells) and not only because of lactogenic hormone-stimulated virus production. The hormonal increase in MMTV transcription may have evolved predominantly to maximize milk production of virus.

Other genes confer resistance to MMTV infection

After the discovery that there was an infectious agent that could be transmitted through milk to nursing pups, it was found that not all strains of mice were equally able to be infected with MMTV. Notably, the C57BL mouse strain and its derivatives were shown to have very low mammary tumor incidence when foster-nursed on C3H/He mice known to transmit virus either to their own pups or to other mouse strains, such as BALB/c[37]. Genetic studies mapped one major resistance gene to the MHC locus in C57BL mice[37] and an additional resistance locus that could be genetically segregated from the MHC locus[38]. This is because C57BL mice and related strains have a genomic deletion of their I-E gene. Most of the MMTV-encoded Sags are only efficiently presented by I-E and not I-A[39]. Because they lack I-E, there is no Sag presentation in C57BL mice and as a result, little or no stimulation of cognate T cells occurs. Hence, MMTV infection is ineffi-

cient. Indeed, it has been shown that C57Bl/6 mice transgenic for I-E are easily infected by milk-borne MMTV (C3H)[40].

C57BL mice contain an additional virus resistance gene. Classical backcrossing studies demonstrated that this resistance locus could be genetically segregated from the MHC locus[38]. These genetic studies have recently been confirmed in our lab using B10.BR mice, which have the same MHC allele as C3H/He (H-2^k) on a C57BL/10 background. However, B10.BR mice are less infected by MMTV(C3H) in comparison with the MMTV-susceptible strain, C3H/HeN, when nursed on the same C3H/HeN MMTV$^+$ mothers (Fig. 3, lanes C3H$^+$ versus B10.BR$^+$). Infection in this case was determined by the amount of viral RNA transcribed in the lactating mammary gland or the amount of virions shed into milk. We also found that B10.BR lymphoid organs (spleen and thymus) have much less exogenous viral DNA than do C3H/HeN mice nursed on viremic mothers (not shown). Backcrosses between C3H/HeN and B10.BR mice indicate that there is a single dominant susceptibility gene in the C3H background (or conversely a single, recessive resistance gene in B10.BR mice) (Fig. 3).

We have done preliminary analyses to map the chromosomal location of this gene, with the goal of identifying it. We have ruled out several candidate loci, including the MHC, endogenous MMTVs and the receptor for the virus, which we have recently cloned in our lab[41]. Thus, the identification of this gene may provide us with new information about how hosts can develop genetic resistance to viral infections.

The lack of the susceptibility gene in the B10.BR has profound effects not only on viral infection, but on tumor induction by MMTV. F2 mice that retain the resistance locus from the B10.BR mice are thus far (> 400 days old) completely tumor-free, while their siblings that inherit the susceptibility gene from the C3H/HeN parent began to develop tumors at 250 days.

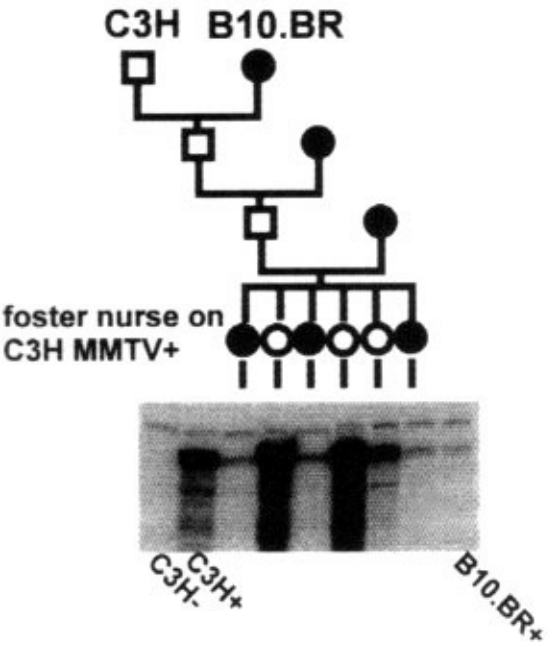

Fig. 3.– B10.BR mice have a recessive gene that makes them resistant to MMTV infection. The indicated crosses were performed and at the N3 generation, the offspring were foster-nursed on C3H/Hen MMTV+ mothers. The offspring were bred and milk isolated after their second pregnancy was subjected to RNase protection analysis specific for MMTV(C3H).
Abbreviations: C3H, milk RNA from an uninfected MMTV(C3H) mouse; C3H+, milk RNA from a C3H/HeN MMTV+ mouse; B10.BR+, milk from a B10.BR mouse foster-nursed on a C3H/HeN MMTV+ female.

Conclusions

The major and perhaps only route of natural infection for MMTV is through milk. Since the mammary gland cell is the ultimate target for this virus, it is not surprising that MMTV acquired the ability to replicate and amplify in cells of the lymphoid system of newborn, nursing pups. The major form of genetic resistance to MMTV identified thus far in mice is the prevention of Sag stimulation of lymphoid cells, thereby blocking infection at the early stages. However, other forms of resistance also exist and these may involve other steps in the infection pathway. For example, efficient virus spread within the mammary gland leading to the insertional activation of oncogenes and tumorigenesis also requires infected lymphocytes and this step could be affected. Similarly, immune recognition of the virus may play a role in the ability of different inbred strains of mice to eliminate or prevent virus infection.

In conclusion, MMTV is a virus that has evolved to take maximum advantage of its host, the mouse. By studying this virus, we not only

learn about the virus life cycle, but about the different aspects of mammalian biology that are used by this virus, including basic transcriptional regulatory mechanisms, the cell biology of the lactating mammary gland and how the immune system functions. We have learned that there is genetic adaptation by the mouse to this virus, such that there is differential susceptibility to infection controlled by genetic background. Understanding the mechanisms of genetic resistance in this model system will lead to increased knowledge about variations in human susceptibility to viral infection and mammary tumorigenesis.

Resumen

El virus del tumor de mama murino (MMTV), un retrovirus que explota el sistema inmune. Genética de la susceptibilidad a la infección por MMTV

Todos los animales, incluso el hombre, presentan diferentes grados de susceptibilidad frente a infecciones inducidas por virus. Los ratones endocriados son los que mejor se prestan al estudio genético de la susceptibilidad o de la resistencia a patógenos específicos. Hemos recurrido al virus del tumor de mama murino, (MMTV), un retrovirus que induce cáncer de mama en ratones, para estudiar la interrelación tumor-huésped. El objetivo era dilucidar los mecanismos que determinan la susceptibilidad genética a tumores de mama inducidos por MMTV, la regulación de la expresión de estos genes in vivo y determinar cómo se transmite el virus entre diferentes células. Hemos encontrado que algunos MMTVs endógenos se expresan solamente en tejido linfoide y que una alteración en un solo par de bases en el LTR del MMTV determina si el virus se expresará o no en la glándula mamaria. Esta expresión génica en células linfoides es necesaria para completar el ciclo infeccioso de MMTV, y tanto las células T como B expresan y secretan MMTV. Se requieren linfocitos infectados no sólo para la llegada inicial de MMTV a la glándula mamaria, pero también para la subsecuente diseminación viral. Faltando esta última, la tumorigénesis mamaria está dramáticamente inhibida. La incidencia de tumores mamarios está también afectada por el *background* genético del ratón y falta identificar por lo menos un gen clave para la infección de ambos linfocitos y células mamarias. Los resultados de nuestros experimentos ayudarán a comprender los mecanismos genéticos que estos virus emplean para infectar sus huéspedes y cómo puede surgir una resistencia genética a estos virus.

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