PRIMARY VIRUS-INDUCED LYMPHOMAS EVADE T CELL IMMUNITY BY FAILURE TO EXPRESS VIRAL ANTIGENS

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It is well established that MHC-restricted T cell responses decisively influence the outcome of virus infection (1-3). In the rejection of murine leukemia virus-induced lymphomas, Th and cytotoxic T (Tc) cells are considered essential effector cells, besides the effects of MuLV-specific antibodies, which can neutralize the virus and inhibit its spread (reviewed in reference 4). Many attempts have been made to characterize the viral antigens that are important in recognition by murine leukemia virus (MuLV)-specific T cells. The presence of viral proteins on the cell surface of MuLV-induced tumor cell lines has been documented in a number of serological studies. These cell surface viral proteins, which in processed form are potential target molecules for recognition by Th and Tc cells (5), include the viral env gene-encoded products gPr85env, gp70, and p15(E) and the gag gene-encoded gP95gag, gP85gag, p10, pp12, p30, and p15 (reviewed in reference 6). In addition to this, recent data indicate that viral proteins that are not detectable at the surface of cells by serology can also serve as targets for T cells (7). DNA-mediated gene transfer experiments, in which single MuLV viral antigens together with particular MHC class I antigens were expressed in heterologous cell lines, and the availability of Tc clones have provided a more subtle analysis of MHC and viral antigen recognition (8, 9). A highly complex picture emerged from these studies, which collectively indicate that both MHC and non-MHC genes determine which viral antigens are recognized in the context of MHC.

Recently, substantial progress has been made in the understanding of MHC-restricted recognition of antigens by T cells. It now appears that T cells usually recognize relatively short antigenic peptides, derived by processing of the native antigen (10, 11). This holds for MHC class II-restricted antigen recognition at the surface of APC by Th cells, but also for MHC class I-restricted antigen recognition by Tc cells, which results in lysis of the antigenic peptide-bearing cells (7, 12). The MHC-peptide interaction required for T cell recognition is rather MHC allele specific, which implies that the nature of viral antigens preferentially recognized differs from individual to individual (7, 13-17).

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Abbreviations used in this paper: BFA, buffered formaldehyde acetone; CFG, cytofluorography; HPS, human pool serum; LTR, long terminal repeat; MoMuLV, Moloney MuLV, MuLV, murine leukemia virus; Tc, cytotoxic T cells.
MuLV-induced lymphomagenesis results from a complex series of oncogenic events in virus-infected cells (18). The development of lymphomas is strongly influenced by the H-2 complex, which probably reflects the fact that virus-infected cells will not grow out to overt lymphomas in the presence of H-2-restricted MuLV-specific Th and Tc responses. However, T cell immunosurveillance will fail if tumor cells do not express the relevant MHC and/or viral peptide at the cell surface. Indeed, various examples have been described of so-called virus-negative variant murine lymphoma cell lines that appeared to be resistant against T cell effector mechanisms. These variants, however, all arose in rather artificial situations, e.g., long-term in vitro culture of tumor cells (19) or under selective pressure of exogenously added Tc therapy (20). The important role of class I MHC gene expression in more natural conditions, i.e., in tumorigenesis and metastasis, follows from a number of studies (21, 22). Low surface expression of class I MHC antigens in MuLV-induced lymphomas has been observed incidentally, e.g., on an AKR thymoma-derived cell line (22), certain radiation leukemia virus-induced lymphomas (23), and Moloney MuLV (MoMuLV)-induced T cell lymphomas (24).

We are interested in the in vivo biological relevance of the proposed selection mechanisms for tumor cells with respect to viral antigen and MHC cell surface expression. We previously described that the H-2 complex strongly influences lymphoma incidence after neonatal infection of C57BL/10 and C57BL/6 mice with mink cell focus-inducing (MCF) MuLV, MCF 1233 virus (25). T cell tumor resistance is dominant, maps to the class II MHC-encoding I-A region, and is associated with high titers of anti-MuLV antibodies, which develop in the presence of an adequate I-A-regulated antiviral Th response. 64% of animals of susceptible, so-called nonresponder strains (H-2 I-A^k,d) developed T cell lymphomas (mean latency, 37 wk). In contrast, only 14% of resistant, so-called responder strains (H-2 I-A^b,bm12,bk) developed T cell lymphomas, the mean latency of which (57 wk) was significantly longer. We decided to compare the antigenic profiles, including both viral antigens and MHC antigens, of the many MCF 1233 MuLV-induced T cell lymphomas that arose in the absence of a MHC class II-regulated immune response with those of the few T cell lymphomas that came up in spite of the presence of a good class II-regulated immune response, reasoning that the latter lymphomas, in contrast to the former, were subjected to strong immunoselective pressure.

Our results show indeed the absence of particular env and/or gag proteins, or more rarely of particular MHC class I antigens on the cell surface of all responder T cell lymphomas tested, in sharp contrast to the abundant expression of both env and gag proteins and MHC antigens on nonresponder T cell lymphomas. Additionally, in a limited study surface, expression differences were confirmed at the RNA level.

Materials and Methods

Mice, Virus, and Lymphomas. Primary lymphomas were induced by injection of newborn mice of C57BL/10 or C57BL/6 background, carrying various H-2 haplotypes as listed in Table I, with MCF 1233 MuLV as described (25). The origin and characteristics of MCF 1233 were described previously (26). This dualtropic B-tropic MuLV was biologically cloned from a T cell tumor, which arose in a B10.A mouse after milk transmission of a naturally occurring B-tropic ecotropic MuLV (27). At autopsy of moribund mice, affected lymphoid organs were removed and handled in three ways. For histologic evaluation, part of the tumor was fixed in formalin (10% in PBS). Part of the tumor was cryopreserved as a viable cell suspension.
in liquid nitrogen in 10% DMSO as described previously (28). The remainder of the tumor tissue was frozen in liquid nitrogen and stored at \(-70^\circ \text{C}\) for DNA and RNA isolation procedures. Lymphomas were classified according to combined phenotypic, histologic, and genotypic criteria as described (29). In vivo passages of lymphomas were carried out by intraperitoneal inoculation of 5-10 \(\times 10^6\) viable cells recovered after thawing of cryopreserved tumor cell suspensions. C57BL/10, B6.C-H-2\(^{14}\) and C57BL/6.KH nude mice used in transplantation experiments were obtained from the animal department of the Netherlands Cancer Institute, Amsterdam.

Immunofluorescence Studies. After thawing, tumor cell suspensions were incubated at 37°C for 2.5 h in Iscove’s modified Dulbecco medium supplemented with 10% heat-inactivated FCS, penicillin (100 \(\mu\)g/ml), kanamycin (100 \(\mu\)g/ml), glutamine (2 mM), and 2-ME (20 \(\mu\)M). To remove nonviable cells and erythrocytes, we centrifuged 2 ml of cell suspension (containing a maximum of 10\(^7\) cells) at 1,000 \(\times \) g for 15 min on a 4-ml cushion of Ficoll-Hypaque (1.079 g/cm\(^3\)). Consistently, >90% of tumor cells recovered this way were viable. In vivo passaged lymphomas were tested either directly ex vivo or after thawing of cryopreserved cells as described above.

Membrane Fluorescence. After washing, cells were resuspended in cytofluorography buffer (CFG buffer: PBS with 2% BSA and 0.1% sodium azide). We used mAbs against Thy-1 (59 AD2.2), Lyt-2 (53-6.7) (30), L3T4 (H129.19) (31), and Pgp-1 (142/5) (32). The following anti-MHC mAbs were used: B.3.24 (anti H-2K\(^b\)) (33), Cl 1.1.4.1 (anti H-2K\(^\text{d}\)) (34), 27-11-13 (anti H-2D\(^\text{d}\), b, bmi4) (35), and 17/227R7 (anti H-2 I-Ab, k.d) (36).

mAb 35/56, a rat IgG2a antibody derived from hybridoma C2346, reacts with the highly conserved gp70 epitope, expressed by ecotropic and MCF MuLV virions, and on ecotropic or MCF MuLV-infected cells (37, 38). mAb 19-F8, a mouse IgG2b antibody derived from hybridoma C2429, reacts with the highly conserved p5(E)\(^1\) epitope, expressed by ecotropic and MCF MuLV virions and on ecotropic or MCF MuLV-infected cells (39). Hy-13, a mouse IgM antibody, reacts with a conformational determinant formed by the gp70-p5(E) complex, and with the ew precursor polyprotein pPr85\(^\text{env}\) of MCF MuLV, which are both expressed on MCF MuLV-infected cells (40). The origins and characteristics of these antiviral mAbs have been described in detail (37-40). The polyclonal goat serum prepared against p30 protein of Rauscher MuLV was obtained from the Viral Oncology Program of the National Cancer Institute (Bethesda, MD). Both ecotropic and MCF gag antigens are detected by this serum, as the gag region is highly conserved between ecotropic and MCF MuLV (reviewed in reference 6).

We incubated 2 \(\times 10^5\) cells with 25 \(\mu\)l antibody for 30 min at 4°C. After washing once in CFG buffer, 25 \(\mu\)l of a second antibody was added, followed by an incubation of 30 min at 4°C. As second antibodies, the following FITC-labeled antisera were used: rabbit anti-rat Ig (1:30; Organon Teknika, Malvern, PA), goat anti-mouse Ig (1:20; Central Laboratory of the Blood Transfusion Service [CLB], Amsterdam), and rabbit anti-goat Ig (1:20; CLB). After two additional washsteps the tumor cells were analyzed for fluorescence on a FACScan (Becton Dickinson & Co., Mountain View, CA) fitted with a four-decades logarithmic amplifier. All cell populations were at least 75% viable as determined by trypan blue uptake and low-angle light scatter at the time of fluorescence. Nonviable cells were gated out.

BFA Fixation of Tumor Cells and Fluorescence Studies on Intracellular Antigens. After washing, 1-5 \(\times 10^5\) cells were resuspended in 1 ml of freshly prepared buffered formaldehyde acetone (BFA) (1.12 mM Na\(_2\)HPO\(_4\), 7.35 mM KH\(_2\)PO\(_4\), 45% vol/vol aceton, 9.25% vol/vol formaldehyde (37%), pH 7.3) for 2 s at room temperature. Subsequently, 5 ml Iscove’s medium supplemented as described above were added. After two wash steps in chilled PBS supplemented with 20% human pool serum (HPS), 2 \(\times 10^5\) cells per sample were stained as described for membrane fluorescence studies, however, 50 \(\mu\)l of antibody supplemented with 20% HPS was used per sample and wash steps were carried out in PBS with 20% HPS. Fluorescence analyses with the FACScan were performed as described for membrane fluorescence analyses.

Removal of Cell Surface Antigens by Trypsin Digestion. Cells were washed once in PBS and subsequently incubated at a concentration of 2 \(\times 10^5\) cells/ml in PBS at 37°C for 5 min. Then an equal volume of trypsin (17.5 U/ml in PBS) was added. Digestion at 37°C was stopped at various timepoints by adding chilled Iscove’s medium supplemented with 10% vol/vol BSA,
penicillin (100 μg/ml), kanamycin (100 μg/ml), glutamine (2 mM), and 2-ME (20 μM). After two washsteps in supplemented Iscove’s medium, the trypsin-treated cells were divided into two portions: cells were stained either for membrane immunofluorescence or, after an additional BFA fixation step, for intracellular fluorescence as described.

**DNA and RNA Analyses.** Cellular DNAs were isolated and analyzed by Southern blotting as described (29). Restriction enzymes were used as described by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN).

For RNA isolation, frozen tissues were homogenized with a polytron (1 min, full speed) at 0°C in 3 M LiCl, 6 M urea, and maintained overnight at 0°C. After centrifugation (4°C, 10,000 g, 60 min) the pellet was dissolved in 2 ml of 10 mM TrisCl, pH 7.4, supplemented with 50 μg/ml proteinase K and 0.5% wt/vol SDS and incubated at 37°C for 30 min. Then, 2 ml of phenol equilibration buffer solution (0.1 M NaCl; 10 mM TrisCl, pH 9.0; 0.5% wt/vol SDS, 5 mM EDTA) were added. Subsequently the RNA was deproteinized by two successive phenol/chloroform/isoamylalcohol (50:48:2) and two chloroform/isoamylalcohol (99:1) extractions and precipitated in 0.3 M NaAc by adding 2.5 vol of ethanol (−20°C). Northern blot analysis of RNA with formaldehyde-treated 0.8% agarose gels was performed as described (21).

Probes were nick translated with 32P-dCTP to specific activities of 7–8 × 10^6 cpm/μg. The isolation and characterization of the MuLV-specific hybridization probes have been described (41–43). Probes corresponding to the middle and COOH-terminal region of p15E of ecotropic AKV-MuLV (MCp15E probe), the U3LTR of ecotropic AKV-MuLV (AKV-LTR probe), the NH2-terminal part of the gp70 region of ecotropic AKV-MuLV (eco gp70 probe), and the NH2-terminal part of the gp70 region of MCF-MuLV (MCF gp70 probe) were used. The MoVl-gag probe, corresponding to the gag region of MoMuLV, was obtained by isolating the 5.3-Kbp KpnI fragment of MoMuLV clone 1a (44; courtesy of Dr. A. Berns, Netherlands Cancer Institute). These MuLV hybridization probes are schematically listed in Fig. 1, in which their ecotropic and/or MCF specificities are given as well.

The γ-actin probe, a hamster cDNA, was a gift of Dr. R. Nusse, Netherlands Cancer Institute. The N-myc probe was a 3.1-kb Pst-1 fragment of the mouse N-myc gene (courtesy of Dr. A. Berns).

TCR β chain probes 86T5, hybridizing to the constant regions, and J15, hybridizing to the Jβ2-Cβ2 intron, have been described previously (29).

### Results

**Viral env and gag Antigen Tumor Cell Surface Expression.** Primary T cell lymphomas of both H-2 I-A' (nonresponder) mice (n = 17) and H-2 I-A'b,bk (responder) mice (n = 11) were analyzed in membrane immunofluorescence experiments for the cell surface expression of MuLV env and gag antigens. We previously documented that the H-2 I-A′ (nonresponder) strains B10.A, B10.A(2R), and B10.A(4R) are equally susceptible to T cell lymphoma induction by neonatal infection with MCF 1233 MuLV (T lymphoma incidence 64%, mean latency 37 wk). H-2 I-A'b,bk (responder) strains B10, B10.A(5R), (B10 × B10.A)F1, B6, bm1, and bm14 are relatively resistant to MCF1233-induced T cell lymphomagenesis (T lymphoma incidence 14%, mean latency 57 wk) (25). In the present study, nonresponder tumors were randomly chosen out of a large panel of nonresponder tumors, whereas all responder tumors that were available have been tested. Tables I and II summarize the data obtained for the two series of lymphomas. A polyclonal goat anti-p30 serum (reactive with p30 and its precursor polyproteins) was used in addition to a panel of mAbs reactive with env proteins: anti-gp70 mAb 35/56, and anti-p15E mAb 19-F8 react with a highly conserved gp70 and p15E epitope, respectively. These epitopes are expressed by both MCF and ecotropic virions and on the cell surface of MCF and ecotropic virus–infected
cells (37–39). The anti-gp70-p15(E) mAb Hy-13 reacts with an epitope formed by the gp70-p15E complex of MCF MuLV (40).

A high percentage (90–100%) of the lymphoma cells of all nonresponder T lymphomas (n = 17), which arose in the absence of a demonstrable antiviral antibody response, showed bright fluorescence with both env- and gag-specific antiviral sera (Table I). All nonresponder T cell lymphomas stained with the anti-gp70<sup>f</sup> mAb (35:56) and the anti-p15(E)<sup>c</sup> mAb (19-F8). Two tumors (2554 and 2594) lacked the expression of the Hy-13-reactive MCF-env determinant. An additional test with a panel of anti-gp70 mAbs (anti-gp70a-e) (39) indicated that these Hy-13- tumors expressed various ecotropic gp70 determinants (data not shown).

In the responder T cell lymphomas (n = 11) a different picture was found (Table II). 6 of 11 tumors tested did not express gag antigens at the cell surface, as concluded from the complete absence of membrane fluorescence after incubation with the polyclonal anti-p30 serum. On one of these six gag-negative lymphomas (2769), hardly any envelope viral antigens were detected; only the anti-gp70<sup>f</sup> mAb was weakly positive (data not shown). One tumor (2498) showed minimal gag expression, and, moreover, lacked the gp70<sup>f</sup> epitope (which was ubiquitously found on the nonresponder lymphomas). Three lymphomas (2875, 2402, and 2810) that did express gag did not express the gp70<sup>f</sup> epitope. The last tumor of this series of responder T cell lymphomas (2558) was the only one to express both gp70<sup>f</sup> env and gag antigens, but, as will be discussed below, this lymphoma lacked H-2 class I K expression.

When tested for the expression of the Hy-13-reactive gp70/p15E determinant, only two responder T cell lymphomas (2498, gp70<sup>f</sup>-negative and 5045, p30-negative) showed bright fluorescence, one tumor (2558) showed dull fluorescence, whereas eight lymphomas did not stain. Staining of responder lymphomas with the p15(E)-
Table I

| Strain       | H-2 type | Viral antigens | MHC antigens |
|--------------|----------|----------------|--------------|
|              | K  I-A  I-E D | envelope pl5(E) gp70p15(E) | Class I K b,d |
|              |          | core p30 | Class II I-A χ |
| B10.A        | k  k  k  d | 2873 3 3 3 3 3 1 1 1 |
|              |          | 2894 3 3 3 3 3 3 3 0 |
|              |          | 3666 3 2 1 2 0 0 0 0 |
|              |          | 3906 3 2 1 2 3 3 1 2 |
| B10.A(4R)    | k  k  b  b | 2456 3 2 2 2 3 3 3 0 |
|              |          | 2522 3 2 3 3 3 3 3 0 |
|              |          | 2877 3 1 1 1 3 3 3 0 |
|              |          | 2534 3 3 0 3 3 3 3 0 |
|              |          | 2985 3 2 3 3 3 3 3 0 |
|              |          | 3817 3 3 3 3 3 3 3 0 |
|              |          | 3846 3 2 2 2 3 3 ND |
|              |          | 3167 2 2 2 2 3 3 ND |
| B10.A(2R)    | k  k  b  b | 2508 3 2 2 2 3 3 3 0 |
|              |          | 2543 3 2 2 2 3 3 3 0 |
|              |          | 2594 3 1 0 3 3 3 ND |
|              |          | 2639 3 3 3 2 3 3 3 0 |
|              |          | 2648 3 3 3 3 3 3 3 0 |

Fluorescence analyses of viral and MHC antigen expression in primary MCF 1233-induced T cell lymphomas of H-2 nonresponder-type mice. Nonresponder H-21-A mice were defined in reference 1.

* All lymphomas stained strongly positive for Thy-1 antigen expression. Both double-positive (L3T4+, Ly-2+) and helper phenotype (L3T4+, Ly-2−) lymphomas were observed.

† The intensity of fluorescence staining of lymphoma cells observed with virus- or MHC-specific antisera (as described in detail in Materials and methods) was related to the intensity of fluorescence observed when control stained. For each individual lymphoma, negative controls constituted lymphoma cells incubated first with indifferent culture supernatant or mouse ascites, followed by a second incubation step with an appropriate FITC-labeled anti-Ig serum. In case of p30 (tested with a polyclonal goat antiserum) normal goat serum was used in the first incubation as a negative control. The observed staining intensity was quantified as follows. Negative control, mean fluorescence intensity μ, standard deviation of the mean s. Fluorescence score for virus or MHC antigen expression: 3, strongly positive; mean fluorescence intensity > μ + 4 s; 2, positive; μ + 2 s < mean fluorescence intensity < μ + 4 s; 1, weakly positive; μ + s < mean fluorescence intensity < μ + 2 s; 0, negative; mean fluorescence intensity < μ + s.

Reactive mAb was somewhat more heterogeneous, but in general, only dull fluorescence was seen when compared with nonresponder T cell lymphomas. Four gp70 f-negative lymphomas were negative or only weakly positive with the anti-p15(E) mAb. Whereas one gp70 f-negative lymphoma (2873) was clearly positive.

Thus, in summary on all 17 nonresponder tumors, abundant gag and env expression was found, whereas 10 of 11 responder tumors lacked gag and/or env determinants on their cell surfaces.

MHC Class I and II Tumor Cell Surface Expression. Cell surface expression of MHC class I (K,D) and class II (I-A) antigens on the T cell lymphomas was determined in immunofluorescence analyses with the use of H-2K, -D, and -I-A-specific mAbs. In the series of (virus expressing) nonresponder T cell tumors, 15 of 17 tumors tested showed high expression of both K and D. One tumor (2873) had weak H-2K and
TABLE II

Decreased Viral Antigen Expression in Primary MCF 1233 MuLV-induced T Cell Lymphomas of H-2 Responder-type Mice: Responder T Lymphomas

| Strain | H-2 type | Viral antigens | MHC antigens |
|--------|----------|----------------|--------------|
|        | K | I-A | I-E | D | Tumor | gp70 | gp70-p15(E) | gp15(E) | gp30 | K^b,k | D^b,d | I-A^b,k |
| B10    | b | b | b | b | 2498 | 0 | 1 | 2 | 1 | 2 | 3 | 0 |
|        | 2729 | 2 | 0^6 | 0^6 | 0^6 | 3 | 3 | 0 |
|        | 2875 | 0 | 2 | 0 | 3 | 3 | 0 |
| B6     | b | b | b | b | 2977 | 3 | 2 | 0 | 0 | 3 | 3 | ND |
| bm1    | bm1 | b | b | b | 2402 | 0 | 0 | 0 | 3 | 2 | 3 | 0 |
| bm14   | b | b | b | bm14 | 2769 | 0^6 | 0^6 | 0^6 | 3 | 3 | 0 |
| B10.A(5R) | b | b | k | d | 2558 | 3 | 2 | 1 | 2 | 0 | 0 | 0 |
|        | 3393 | 3 | 1 | 0 | 0 | 0 | 3 |
| (B10.A x B10)F1 | k/b | k/b | k/b | k/b | 2810 | 0 | 1 | 0 | 3 | 2 | 3 | 0 |
| (B10 x B10.A)F1 | b/k | b/k | b/k | b/k | 3915 | 3 | 0 | 0 | 0 | 3 | 0 |
|        | 5045 | 2 | 2 | 3 | 0 | 3 | 0 |

Fluorescence analyses of viral and MHC antigen expression in primary MCF 1233-induced T cell lymphomas of H-2I-A responder-type mice. Responder H-2I-A mice were defined in reference 1.

* All lymphomas stained strongly positive for Thy-1 antigen expression. Both double-positive (L3T4⁺, Ly-2⁺) and helper phenotype (L3T4⁺, Ly-2⁻) lymphomas were observed.

† The intensity of fluorescence staining of lymphoma cells observed with virus- or MHC-specific antisera (as described in detail in Materials and Methods) was related to the intensity of fluorescence observed when control stained. For each individual lymphoma, negative controls constituted lymphoma cells incubated first with indifferrent culture supernatant or mouse ascites, followed by a second incubation step with an appropriate FITC-labeled anti-Ig serum. In case of p30 (tested with a polyclonal goat antiserum) normal goat serum was used in the first incubation as a negative control. The observed staining intensity was quantified as follows. Negative control, mean fluorescence intensity μ, standard deviation of the mean s. Fluorescence score for virus or MHC antigen expression: 3, strongly positive; mean fluorescence intensity > μ + 4 s; 2, positive; μ + 2 s < mean fluorescence intensity < μ + 4 s; 1, weakly positive; μ + s < mean fluorescence intensity < μ + 2 s; 0, negative; mean fluorescence intensity < μ + s.

§ Fluorescence analyses have been performed on BFA-fixed cells as well, thus additionally indicating the absence of viral antigen intracellularly.

H-2D expression, whereas another tumor (3666) had minimal if any detectable H-2K and -D antigens. When tested for the expression of Pgp-1, an antigen that is assumed to be related with the IFN-γ producing potential of T cells (45), these very two tumors showed dull Pgp-1 expression in contrast to the 15 high H-2K- and D-expressing lymphomas, which with one exception (2648) had bright Pgp-1 reactivity (data not shown). In the series of responder T cell lymphomas, also, the majority (9 of 11 tumors tested) had high H-2K and -D expression. As already mentioned above, one tumor (2558) that, by exception in the responder series, expressed both env and gag viral antigens at the membrane, had no detectable H-2K^b cells surface antigen, whereas H-2D^d expression was relatively low. One other lymphoma (3993) did not show H-2K^b expression but had high D^d expression. This latter K^b⁻/D^d⁺ tumor showed bright Pgp-1 expression whereas tumor 2558 (K^b⁻/D^d⁺) had a relatively dull Pgp-1 expression (data not shown).

None of the T cell tumors in either series, nonresponder and responder, expressed significant levels of H-2 class II I-A molecules.


**Intracellular Expression of Viral Proteins.** We tested a small number of lymphomas for the intracellular presence of viral proteins in an immunofluorescence assay, by incubation of BFA-fixed lymphoma cells with a selected panel of the aforementioned antiviral sera.

First, we showed that, as illustrated for p30-gag in Fig. 2, after cleavage of the protein of interest at the membrane by trypsin, the intracytoplasmic viral antigen remained detectable. With this method no discrepancies were found between the cell surface and intracellular expression of anti-p30-, anti-p15E', anti-gp70/p15E-, and anti-gp70'-reactive viral proteins in tumors 2729, 2769, and 2522 (Tables I and II). Thus, the cell surface expression of viral antigens correlated strictly with the presence or absence of these viral antigens intracellularly.

![Intracellular fluorescence of BFA-fixed tumor cells after removal of p30 viral antigen from the cell surface by trypsin exposure.](image)

**Figure 2.** Intracellular fluorescence of BFA-fixed tumor cells after removal of p30 viral antigen from the cell surface by trypsin exposure. Lymphoma cells (2522, nonresponder type) were analyzed in indirect fluorescence staining for p30 antigen expression by incubation with anti-p30-reactive goat serum, followed by FITC-labeled anti-goat Ig serum. Dotted lines represent negative control-stained cells (first incubation step with normal goat serum). (I) Cells before trypsin treatment; (IA) membrane fluorescence; (IB) intracellular fluorescence of BFA-fixed cells. (II) Cells after 15 min of trypsin (17.5 U/ml) exposure; (II A) membrane fluorescence; (II B) intracellular fluorescence of subsequently BFA-fixed cells.
Proviral Integrations. T lymphomas of nonresponder and responder mice were analyzed for the presence of newly acquired proviruses. Lymphoma DNAs were cut with Eco RI, which has no restriction site in a full-length ecotropic MuLV provirus and a single site in MCF MuLV at 6.9 kb (46). Hybridizations were carried out with the use of an (MCF and eco-reactive) U3LTR probe and an eco-specific gp70 probe (Fig. 1). The results (Fig. 3) indicate that both nonresponder and responder

![Figure 3](image-url)
T cell lymphoma DNAs contained multiple, predominantly MCF-like proviruses. A few exceptions were observed; e.g., tumor 2729 seems to contain only one extra clonal fragment (~20 kb) reactive with both MuLV probes, indicating its ectropic origin. In search of possible oncogene rearrangements due to proviral integrations, we hybridized EcoRI-digested lymphoma DNAs with a panel of oncogene probes; we found no c-myc rearrangements, but in two nonresponder lymphomas (2894 and 3666) and three responder lymphomas (2402, 2498, and 2875), pim-1 rearrangements were found (data not shown). N-myc rearrangements were detected in one nonresponder tumor (3846) (data not shown) and two responder tumors (2729 and 2997) (Fig. 3B). The pim-1- and N-myc-reactive rearranged fragments comigrated with U3LTR-reactive DNA fragments, probably indicating that the oncogene rearrangements were due to proviral integrations nearby.

RNA Analyses. To reveal whether the absence of particular viral antigens at the cell surface of responder T cell lymphomas was correlated with quantitative or qualitative changes in viral RNA species found in the tumor cells, we performed Northern blot analyses for a small number of lymphomas (Fig. 4). We had to make use of first transplant generation tumors, because primary tumor tissues had not been conserved adequately for RNA isolation procedures. The phenotypes of these syngeneic in vivo passaged lymphomas remained completely stable (shown for 2769 in Fig. 5). Various probes hybridizing to U3LTR, p53E, and gp70, and gag sequences of MCF and/or ectropic origin (Fig. 1) were used. Four nonresponder T cell lymphomas, bearing gag and env antigen at their cell surfaces, showed high amounts of viral RNAs of the expected sizes: 8.2-kb genomic RNA, from which the gag and pol products are ultimately derived and 3.2-kb spliced mRNA, which yields the env products (6). In contrast, only minimal amounts of both 8.2- and 3.2-kb RNAs were found in two non responder T cell lymphomas, in various individual transplants.

In tumor 2729, which at the cell surface had moderate env, but no detectable gag expression, an aberrant RNA of 2.5 kb was found, which hybridized only to the U3LTR probe but not to (eco- and MCF-specific) p53(E), gp70, and gag probes (Fig. 4; gag hybridization shown in Fig. 6). In this tumor a rearrangement of the N-myc gene, due to proviral integration, had been found (Fig. 3 B). Reasoning that this proviral integration might influence N-myc transcription, we hybridized 2729 RNA with the N-myc probe (Fig. 4 B). Indeed, the U3LTR-reactive 2.5-kb RNA in tumor 2729 hybridized also to the N-myc probe. In tumor 2759, on the cell surface of which no gag and only weak gp70 env antigens were detected, an aberrant RNA (4 kb) was found as well; this RNA hybridized to both the U3LTR and p53(E) probes, but not to gp70 env and gag probes (Fig. 4; gag hybridization shown in Fig. 6). Thus, both quantitative and qualitative differences in viral RNAs were observed when we compared nonresponder and responder lymphomas. The absence of cell surface viral antigen expression correlated with low amounts of normal viral RNA species.

Involvement of T Cell Reactivity. The findings thus far strongly suggested that the absence of particular env and/or gag proteins on the tumor cell surface is only required in the face of a class II-regulated T cell response in H-2 I-A responder type mice. Therefore, it seemed of interest to determine whether the "virus-negative" variant T cell tumors would re-express env and/or gag proteins if transplanted in T cell-deficient nu/nu mice. We transplanted two responder T cell tumors, tumor 2729 and 2769, in C57BL/6 nu/nu mice, and studied virus expression in the transplants at the pro-
Figure 4. (A) Expression of viral RNAs in nonresponder and responder type T cell lymphomas induced by MCF1233. RNAs were isolated from the first in vivo syngeneic passages of four nonresponder (lanes 1-4) and two responder (lanes 5-7) lymphomas. Northern blot analysis of 20 μg total cellular RNA on formaldehyde-treated 0.8% agarose gels was performed as described (21). The nitrocellulose filter was successively incubated with U3LTR (A), γ actin (E), MCpl5E (B), eco gp70 (C), and MCF gp70 (D) specific hybridization probes. Rehybridizations were preceded by washing the filter in T10EI (10 mM Tris, 1 mM EDTA) at 80°C for 1 h.

RNAs were obtained from first syngeneic transplants of: lane 1, tumor 2873 (B10.A); lane 2, tumor 2543 (B10.A(2R)); lane 3, tumor 2639 (B10.A(2R)); lane 4, tumor 2554 (B10.A(4R)); lanes 5 and 6, tumor 2729 (B10), two individual transplants (lane 5, mouse Ia, lane 6, mouse Ib); lane 7, tumor 2769 (bm 14), mouse Ia. RNA of the other first transplant of tumor 2769 (mouse Ib) gave the same picture upon hybridization to the various MuLV-specific probes as shown in lane 7 for RNA of the transplant in mouse Ia (data not shown). The characteristics of viral and MHC antigen cell surface expression on the first syngeneic transplants were identical to those of the primary lymphomas, which are given in Table I (nonresponders, lanes 1-4) and Table II (responders, lanes 5-7). (B) N-myc containing viral RNA in tumor 2729. On a separate gel another 20 μg total cellular RNA, isolated from the two individual syngeneic transplants of tumor 2729 (lanes 5 and 6, in A) were loaded. After blotting, the filter was hybridized to the N-myc probe; lane 5, 2729, mouse Ia; lane 6, 2729, mouse Ib.

Importantly, all individual transplants of 2729 and 2769 showed the same clonal
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A primary responder T lymphoma 2769
B syngeneic passage
C nu/nu passage

FIGURE 5. Viral and MHC antigen cell surface expression on responder T lymphoma 2769. Experimental procedures as outlined in legend to Fig. 2. Horizontal axis, fluorescence intensity (log scale, arbitrary units); vertical axis, cell number. The dotted lines represent the fluorescence profile of the negative control, the solid lines represent tumor cells incubated with a specific antivirus or anti H-2 antibody, as indicated at the left. (A) Primary responder T lymphoma 2769 (bm14); (B) syngeneic passage of 2769; 2769 (Ia); (C) passage of 2769 (Ia) in C57BL/6 nu/nu mouse.
FIGURE 6. Expression of viral RNAs by MCF 1233–induced T cell lymphomas upon passage in immunodeficient C57BL/6 nu/nu mice. Northern blot analysis of 20 μg total cellular RNA on formaldehyde-treated 0.8% agarose gels was performed as described (21). The nitrocellulose filter was successively incubated with U3LTR (A) g actin (C), and MoV1-gag (B) specific hybridization probes. Rehybridizations were preceded by washing the filter in T0El (10 mM Tris, 1 mM EDTA) at 80°C for 1 h. RNAs were isolated from lanes 1 and 2 (negative controls): normal spleen (lane 1), normal thymus (lane 2); lanes 3 and 4 (positive controls): first syngeneic transplant of tumor 2554 (B10.A[4R]) (lane 3), first syngeneic transplant of tumor 2639 (B10.A[2R]) (lane 4); lane 5, first syngeneic transplant of tumor 2729 (B10), mouse Ib; lane 6, passage of tumor 2729 Ib into a C57BL/6 nu/nu mouse; lane 7, first syngeneic transplant of tumor 2769 (bm14), mouse Ia; lanes 8 and 9, two individual transplants of tumor 2769 Ia into C57BL/6 nu/nu mice. Characteristics of viral and MHC antigen cell surface expression on the primary tumors 2554 and 2639 are given in Table I, on the primary tumor 2729 in Table II. The antigenic profiles of the in vivo passaged tumors used for RNA analysis were identical to that of the primary tumors. Analyses of viral and MHC antigen cell surface expression on primary tumor 2769 and subsequent passages into syngeneic and C57BL/6 nu/nu mice are shown in Fig. 5.

TCR β chain gene rearrangement as the parental tumor cells. Moreover, the proviral integration patterns of the primary tumors were identical to those of the transplants for lymphomas 2729 and 2769, passaged in either immunocompetent or nu/nu mice (data not shown).

Discussion

In this study we have documented a major mechanism by which primary immunogenic MuLV-induced T cell tumors can be envisaged to evade T cell immunosurveillance, namely by failure to express particular viral antigens at the tumor cell sur-
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face. More rarely, the absence of class I MHC antigens was observed. Absent viral or MHC class I antigen expression on tumor cells appears only necessary for tumor survival in the face of a strongly Th-dependent antiviral immune response. This conclusion follows from a detailed comparison of viral env/gag and MHC class I antigen cell surface expression in MCF 1233 MuLV-induced T cell lymphomas in H-2 I-A responder and H-2 I-A nonresponder mice.

The absence of p30-reactive cells in 6 of 11 lymphomas of the responder group probably reflects the complete absence of any gag proteins in these lymphoma cells, since the anti-p30 polyclonal serum used reacts with both p30 and its precursor polyproteins (own unpublished results). In view of the well-known heterogeneity of viral gp70 antigens (47, 48) and the reactivity of polyclonal anti-gp70 sera with gp70-like determinants or normal lymphoid cells (49), the use of mAb anti-gp70f (reactive with a highly conserved gp70 epitope) is most appropriate for the comparative serological analyses of gp70-env expression on responder and nonresponder lymphomas.

It should be stressed that in the protection against early T cell lymphomas, H-2 I-A-restricted Th may exert both direct effects, such as the production of lymphokines (e.g., IFN-γ), and indirect effects, such as the production of anti-MuLV antibodies by B cells and/or the augmentation of MuLV-specific Tc activity. We have documented previously that responder mice, in contrast to nonresponder mice, develop high titers of anti-MuLV antibodies (25). The antibody response to MuLV is known to be directed primarily at the envelope proteins gp70 and p15(E) (50). Thus, antibody-dependent cellular effector mechanisms in responder mice will probably fail in the absence of these viral antigens. In addition, data of Nowinski et al. (37), who reported that the anti-gp70f mAb was the only one among the panel anti-gp15 mAbs that neutralized MuLV, may indicate that selectively the gp70f determinant is an important envelope epitope in the humoral anti-MCF 1233 immune response.

Although we have no direct evidence for a protective role of MCF1233-specific Tc activity, in view of many other reports (3, summarized in reference 4), it seems very likely that augmentation of Tc responses by the H-2 I-A-restricted anti-MCF1233 Th response will contribute to the eradication of MCF1233-infected cells. Obviously, no reliable quantification of the amount of viral antigen relevant for T cell immunity can be based on serological membrane fluorescence studies. However, it can be envisaged that, if viral proteins are not detectable serologically, no viral env and/or gag proteins will be available for processing into viral peptides that can be recognized by T cells. Importantly, as discussed below, differences in viral antigen expression detected by serological methods correlated with viral RNA levels in responder and nonresponder lymphomas.

We did not perform immunoprecipitation studies for the quantification of viral proteins for several reasons. First, primary lymphomas would have to be kept in culture, thus, theoretically creating the opportunity for the occurrence of in vitro phenomena such as reexpression of transcriptionally silent proviral genes, which is a real danger in view of data of several groups (6, 20). Furthermore, membrane fluorescence studies may reveal changes in viral antigen expression that are of importance to antiviral immunity, which cannot be detected by methods in which the natural configuration of viral antigens in the cell membrane is first destroyed by the use of detergents.

As stated already, both env-negative and gag-negative variants are detected among
responder T cell lymphomas, but there seems to be no preferential loss of a particular antigen in relation to H-2K or -D allelic specificities. This is in line with data of Plata et al. (9), who demonstrated the presence of both *gag* and *env* AKR Gross MuLV-specific Tc in C57BL/6 (H-2<sup>b</sup>) mice. Moreover, the frequency of *gag* and *env*-specific Tc varied within a given immunized mouse strain (F. Plata, personal communication). Pala et al. (51) reported that the frequency of influenza nucleoprotein-specific Tc varied between individuals of an inbred mouse strain. The immune response against MCF 1233 in H-2 I-A<sup>b</sup> mice will be polyclonal but probably is biased to particular *gag* or *env* antigens, dependent on the immunodominance of these antigens in the original presentation to class II-restricted Th. Thus, loss of *env* or *gag* expression can contribute to a relative, but not an absolute, growth advantage for a pre-leukemic cell.

Other reports of MuLV-induced tumors, which lack viral antigen expression, indicate various immunorelevant target molecules (20, 52-54). It should be stressed that our observations concerning viral antigen and MHC expression on lymphomas were made on fresh or cryopreserved viable cell suspensions from primary lymphomas that arose in vivo in nonmanipulated animals. The few previous observations of absent viral antigen expression on retrovirus-induced lymphomas were made with in vitro tumor lines (52-54), or after artificial immunoselective pressure (20). Green and Manjunath and colleagues (52, 53) described two variant subclones of a tumor cell line, derived from an AKR virus-induced lymphoma in AKR (H-2<sup>k</sup>) mice, that were resistant to lysis by virus-specific Tc. These variants showed strongly reduced gp70 *env* viral antigen expression as detected by the gp70-specific mAb panel, also used here. In line with our data, Green (52) observed selective loss of particular gp70 epitopes. Schäfer and Schmidt (54) documented qualitative and quantitative differences in antigenic *gag* determinants at the cell surface of various AKR thymoma cell lines. Van der Hoorn et al. (20) characterized Tc-resistant antigen loss tumor cell variants that had lost the gp85<sup>MoMuLV</sup> surface antigen but retained transcriptionally silent MoMuLV proviruses. These variants arose from a parental MoMuLV-induced tumor cell line upon transplantation in the presence of exogenously added immunoselective pressure, namely the intravenous inoculation of cloned MoMuLV-specific Tc.

Another study to be discussed in relationship to our data is that of Wolfe et al. (55), in which the infectious virus production in vitro of Friend MuLV-induced tumor cell lines depended on their H-2 types. Remarkably, for unstated reasons, the viral replication grade decreased in vitro in the absence of immune effector function. Although we can not exclude that such a phenomenon also occurs in vivo in MCF 1233-infected H-2 I-A<sup>b</sup> responder type cells, this is unlikely in view of previous results. We have described earlier that MCF 1233 MuLV initially infects thymocytes in both responder and nonresponder mice, as tested by the cell surface expression of Hy-13-reactive *env* viral proteins. However, in responder mice virus-infected cells disappear from the thymus, coinciding with the appearance of anti-MuLV antibodies, the production of which requires an adequate class II-regulated Th response. This observation has prompted us to hypothesize that T cell immune reactivity in H-2I-A<sup>b</sup> mice directly or indirectly clears the viral infection of thymocytes (25).

The significance of low or absent H-2K and -D expression in two nonresponder T cell lymphomas is not clear. It cannot be excluded that helper-independent Tc
may play a minor protective role in H-2I-A nonresponder type mice (25, 56). We describe here one MCF 1233-induced T cell lymphoma (2558) in the responder series that shows selective loss of K\textsuperscript{b} expression with only weak D\textsuperscript{d} expression. Remarkably, this tumor was the only exception in the responder series with respect to the expression of viral env and gag proteins, which were easily detectable in this tumor. Another lymphoma, 3933, lacked K\textsuperscript{b} expression and showed normal env but absent gag expression. We previously showed that the immune response against MCF 1233 is primarily I-A-regulated Th dependent (25). The relative contribution of class I-restricted Tc responses in the eradication of virus-infected cells has not yet been clarified. Interestingly, the exceptional switch off of class I H-2K expression was observed in both cases for B10.A(5R) lymphomas (H-2K\textsuperscript{b}, -I-\textsuperscript{a}, -D\textsuperscript{d}). At this moment it remains speculative whether this observation indicates an important role for K\textsuperscript{b} as sole restriction element if no D\textsuperscript{b} is available. Plata et al. (9) demonstrated that B6 mice recognize both gag and env gene products of Gross (AKR type) MuLV efficiently in association with K\textsuperscript{b} or D\textsuperscript{b}.

It seemed of interest to determine at which level the absence of particular viral proteins at the cell membrane of responder T lymphoma cells was established. To this end, we studied two responder T cell lymphomas (2729 and 2769) in more detail. The direct serological measurement of the intracellular presence of viral proteins after fixation in BFA of the T lymphoma cells did not show discrepancies between membrane and intracytoplasmic expression.

In T cell lymphoma DNAs of both nonresponder mice and responder mice (Fig. 3), multiple newly acquired proviruses could be detected with an U3LTR probe. However, it should be noted that this does not take into account whether or not these integrations represent full-length, partially deleted, or defective proviruses. Insertional mutagenesis, a well-documented oncogenic mechanism in MuLV-induced T cell lymphomas (57-59), only requires the activity of proviral LTR sequences (60). More extensive analyses of the nature of the viral integrations in nonresponder and responder lymphomas is hampered by the presence of multiple MCF- and xenotropic-like endogenous viral sequences (6, 61).

The amount of viral RNAs (8.2 and 3.2 kb) in two responder T cell lymphomas (2729 and 2769) was only minimal when compared with that in four nonresponder T cell lymphomas. The expression of proviruses is known to be influenced by a number of factors. A causal relationship between hypermethylation and gene inactivity has been established for retroviral genomes (20, 62). Moreover the host integration site and specific sequences within the long terminal repeat (LTR) may regulate the viral expression (6). One possibility to be considered in our study is that in H-2I-A responder type mice, only tumor cells that harbor proviruses that do not yield immunorelevant viral proteins but have retained transformational capacities due to insertional mutagenic effects of integratedLTRs will grow out. In this respect, it is of note that the mean latency of T cell tumors in the responder strains (57 wk) is significantly longer than in the nonresponder mice (37 wk) (25). This may reflect the time needed to require the extra and rare conditions required to grow out in the presence of an antiviral immune response. In both responder tumors (2729 and 2769) abnormally sized viral RNAs are found. In tumor 2729 the single proviral integration near N-my\textit{c} apparently results in the expression of a 2.5-kb RNA, containing U3LTR and N-my\textit{c} sequences. It is possible that LTR-enhanced transcrip-
tion of N-myc interferes with transcription of proviral DNA, thus explaining decreased virus expression in tumor 2729. The origin of the aberrant viral RNA found in tumor 2769 remains to be determined.

The stable virus-negative phenotype of tumor 2729 upon in vivo passage in T cell-deficient nu/nu mice is compatible with interference of N-myc and provirus transcription within this tumor, as stated above. However, tumor 2769 re-expressed both viral env and gag proteins upon transplantation in nu/nu mice. Importantly, at the DNA level no obvious changes could be detected with respect to viral integration pattern in the various transplants. This argues against the outgrowth of a pre-existing minor subpopulation of virus-positive cells. Obviously, no general conclusions can be drawn from the limited transplantation data of tumor 2769; more experiments are now in progress to determine how generally the seesaw phenomenon occurs in the absence or presence of T cell immunity. At this moment it is merely speculative which mechanism(s) underlie the re-expression of viral antigens in lymphoma 2769. Noteworthy, no significant differences in macroscopic aspect and/or latency (4 wk) of the transplants were observed, whether they grew out in immunocompetent or deficient mice. One possibility is that in immunocompetent mice, MuLV-specific Th produce IFN-γ, which is known to downregulate the expression of proviral genes (63).

It should be mentioned that we found no strict direct quantitative relationship between the amounts of viral RNA and the presence of particular viral antigens at the tumor cell surface, detected serologically. Tumor 2429 does express gp70 epitopes, whereas only a minimal amount of env probe-reactive RNAs is present. The data discussed thus far indicate that both quantitative and more subtle qualitative changes in viral antigen expression are found in distinct responder T cell tumors.

Our observations on MuLV-induced lymphomagenesis may have important implications for the understanding of the pathogenesis of human virus-induced disease. A study of Massuci et al. (64) shows that certain Burkitt lymphoma cell lines that are resistant to EBV-specific Tc have selectively lost expression of EBV-encoded latent membrane protein, in addition to loss of HLA-A11 expression, suggesting that this mechanism of low viral antigen and/or MHC expression may also operate in man. It is tempting to draw a parallel between our results and those of two recent studies (65, 66), which show that progression of disease in individuals infected with HIV-1 is correlated with the emergence of HIV-1 variants that are more cytopathic and replicate to a higher titer in a wide variety of different human cells. It has been suggested that the infectivity of different HIV virus isolates is established at the transcriptional level. In our view, and as is illustrated by our results, it could well be that these HIV variants emerge as a consequence of the dynamic molecular genetic features of retroviral infection and its close interaction with the host-determined immune response.

Summary

T lymphoma induction by the mink cell focus-inducing murine leukemia virus MCF 1233 in C57BL/10 and C57BL/6 mice is influenced by a strongly Th-dependent, H-2I-A-restricted antiviral immune response (25). We compared the MHC class I as well as viral env and gag antigenic cell surface profiles of frequent T lymphomas of H-2I-A nonresponder-type mice to that of rare T lymphomas of H-2I-A responder-
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type mice. Membrane immunofluorescence studies, with a panel of anti-env mAbs (reactive with the highly conserved gp70' epitope, the p15E' epitope, and the gp70-p15E complex), a polyclonal anti-p30 serum, and anti-H-2 class I mAbs, showed that all 17 nonresponder tumors tested expressed high levels of both env and gag viral proteins, and 15 of these 17 nonresponder tumors expressed high levels of H-2 class I K and D antigens. In contrast, 10 of 11 responder lymphomas lacked env and/or gag determinants. The only responder lymphoma with both strong env and gag expression failed to express H-2K and -D antigens. Preferential loss of env or gag expression did not correlate with H-2 class I allelic specificities.

Both responder and nonresponder T lymphoma DNA contained multiple, predominantly MCF-like, newly acquired proviral integrations. Differences in viral antigen cell surface expression were confirmed at cytoplasmic and RNA levels. The amounts of 8.2- and 3.2-kb viral RNA were greatly reduced in two responder lymphomas when compared with four nonresponder lymphomas. In both responder lymphomas, aberrantly sized viral RNA species were found. Upon in vivo passage of these responder lymphomas in either immunocompetent or T cell-deficient nu/nu mice, it was found that various molecular mechanisms may underlie the lack of viral antigen expression at the cell surface of these lymphomas. One lymphoma re-expressed viral antigens when transplanted with nu/nu mice, whereas the other remained stably gag negative. The combined findings indicate that an H-2I-A-regulated antiviral immune response not only strongly reduces T lymphoma incidence, but also forces T lymphomas that still arise to poorly express viral antigens, thus explaining their escape from immunosurveillance.

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