Evidence in Favor of the Existence of Human Breast Cancer Virus

Dan H. Moore
Institute for Medical Research, Camden, New Jersey 08103

Summary

The existence in some women of a virus that is related to the mouse mammary tumor virus is evidenced by the following. Type B particles are occasionally found in human milk. A complication is the fact that most human milks destroy or damage the murine, mammary tumor virus (MuMTV) and its RNA-directed DNA polymerase (RDDP) when the mouse virus is added to the human milk. Human milk presumably has the same effect on any human RNA virions present. RDDP is found in many human milks although because of the destroying factors (not present in mouse or cow's milk), its presence usually depends on the freshness and/or amount of destroying factors in human milk; RDDP is associated with particles having the same buoyant density as MuMTV (1.18 to 1.22 g/ml versus 1.15 to 1.17 g/ml for leukemia viruses). RDDP is associated with a 35 S or 70 S RNA which is a characteristic of RNA tumor viruses. Human RDDP responds to magnesium ions for activity with a synthetic template, polyribocytidylic-deoxyguanylic (12 to 18 nucleotides long), in agreement with MuMTV probes. RDDP is not absorbed by RIII milk, but was not absorbed by MTV-free monkey virus probes. Using the migration inhibition factor test, positive responses of human leukocytes to homologous in situ breast cancer tissue are correlated with responsiveness to RDDP. Many human sera completely neutralize MuMTV. (The neutralizing activity resided in the globulin fraction, was absorbed by RIII milk, but was not absorbed by MTV-free C57BL milk.) Some human breast cancer patients' sera contain material that precipitates specifically on the membrane of budding MuMTV virions as demonstrated with peroxidase-coupled anti-human globulin. Slices of mouse tumors rich in MuMTV react, in immunofluorescence tests, with sera from some women with breast cancer or fibrocystic mastopathy.

Viruses as etiological agents in cancer have recently received much attention and the etiological relationship of several viruses with specific cancers in various animal and plant species has been established. The question as to whether a virus can cause cancer in man, however, remains unanswered. This is not surprising because oncogenic viruses are not easily recognizable environmental factors; they are difficult to find and identify. The extensively studied oncornaviruses fall into 2 categories, those that cause leukemias and sarcomas in many species and those that cause mammary tumors in mice. Means are now available for identifying oncornaviruses in any species including man and for distinguishing the leukemia-sarcoma type C virions from the mammary tumor type B virions. This article is written in the light of the accumulated evidence that there is a virus in humans with properties that, in general, parallel those of the type B particles of the MuMTV.

Most of the effort to find a human breast cancer virus has taken place during the 4 or 5 years, although searches for virions in human milk and tumors with the electron microscope had already been reported (15, 18, 22, 33, 47). We are not yet sure about the significance of the various particles found in thin-section electron microscopy, but in a negative stain some have every characteristic of MuMTV, i.e., head and tail forms with heads 100 nm in diameter and tails 300 nm long and with the surface covered with 10-nm "spikes" spaced at about 7 nm with knobs at the distal ends 3 to 5 nm in diameter. Others appear to be the same particles damaged to varying degrees. Still others look like the PMPV (3, 12) and others like the typical type C particles associated with leukemia and sarcoma in several species. Although the B particles are very difficult to find in human milk, their presence constituted the first evidence for the hypothesis of a putative human mammary tumor virus (26, 30, 38, 47). Their unique surface characteristics make well-preserved B particles positively identifiable (37, 40). The high-mammary-tumor-mouse strains such as RII have 10^12 particles/ml in their milk whereas most human milks have a concentration of less than 10^8 particles/ml which, even after being concentrated, is at the

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2 The abbreviations used are: MuMTV, murine mammary tumor virus; PMPV, Mason-Pfizer monkey virus; RDDP, RNA-directed DNA polymerase; poly(cC), synthetic polyribocytidylic; oligo(dC), synthetic deoxyguanylic (12 to 18 nucleotides long); MTV, mammary tumor virus; RLV, Rauscher leukemia virus; R35, virus found in a rat mammary tumor.
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limit of detectability in negatively stained dispersions. Possible detection of virions at lower concentrations is attained by thin-section or agar sedimentation procedures (24), but reliable identification of B particles may be less certain (37, 38, 40). Many specimens contained profiles in sections that resembled B particles whereas portions of the same specimen examined by negative stain showed only vesicles either with no surface spikes or with spikes having non-B particle characteristics. In examined milk specimens from more than 1000 women, only 16 uninjured B particles were found by the negative stain procedure.

Virions are unstable in human milk (16, 36). In the process of trying to improve the procedure for isolating virus-like particles, mouse B particles were added to specimens of human milk, and then different methods were used to recover them. Destructive effects were observed readily; B particles were found in various stages of degradation and bioactivity was greatly reduced. Despite this, RDDP was found in some human milks (45) and a procedure was devised for the simultaneous detection of RDDP and its association with high-molecular-weight RNA (35 S or 60 to 70 S) (44, 46). The presence of RDDP and 35 S or 70 S RNA was indicative of an oncorna-
virus in human milk but did not define its exact nature.

In December 1972, a conference on the molecular biology of human milk resulted in the following conclusions (50): (a) RDDP and 60 and 70 S RNA are being found in human milk particles with a buoyant density in sucrose of 1.16 to 1.19 g/ml by all groups making such studies; (b) the particles isolated from some human milks were found to contain polyadenylate sequences in their RNA molecules, a characteristic of tumor virus RNA (42); (c) some human milks mixed with MuMTV caused severe loss of infectivity and morphological destruction of the virions (36). There was also a decrease in RDDP activities of MuMTV, avian myeloblastosis virus and murine leukemia virus when these viruses were mixed with human milks. This decrease in RDDP could be correlated with the amount of RNase found in the milk; (d) unclassified virus-like particles as well as type C and occasionally type B particles are found in human milk samples, which also contain a virulcytic factor or factors in varying amounts. Correlation between family history of breast cancer and the presence of virus-like particles or reverse transcriptase in human milk samples is poor. Neither particles nor RDDP are restricted to members of high-breast-cancer families. Methods of preserving and separating the various types of particles are rapidly improving; (e) the elution profile of a polydeoxythymidylate synthetase from human milk particles on phosphocellulose columns was found to be strikingly different from that of known oncorna-
viruses.

Destruction of Virions by Human Milk

Although all of the 12 laboratories represented at the conference agreed that human milks contained 35 and/or 70 S RNA and RDDP, there was no agreement on the incidence of their occurrence. Some laboratories found them in only 15 to 20% of the specimens while others found them in 75 to 85%. Obviously, there were analytical difficulties (35). Some of these difficulties have been overcome by the introduction of the exogenous template poly(rC)-oligo(dG)12-18 (23, 48) which is very sensitive to the RDDP and thus far has been found to be specific for viral enzyme; nonviral cellular polymerases do not utilize this template primer (21, 23, 48).

Another problem was the instability of RDDP in human milk. This was not limited to the enzyme of endogenous virus but included the RDDP of added MuMTV as well as avian myeloblastosis virus and murine leukemia virus (36, 50). The infectivity of Japanese B encephalitis virus and Friend leukemia virus was also essentially destroyed by human milk (16). RNase, present in widely varying amounts in human milks, was found to be one of the factors that interfered with RDDP determinations. However, since RNase did not act on intact, whole virions, there were other factors that degraded the particles and caused loss of infectivity. Despite the fact that there was no correlation between the detection of B particles in density gradient isolates and the presence of 35 or 70 S RNA and RDDP, the location of human milk RDDP in density gradients, as we shall see later, corresponded to the density of B particles of MuMTV rather than the C particles of mouse leukemia. Why did the buoyant density at which the RDDP was found correspond to that of B particles and yet the virus particles consistently could not be found? The explanation might be that the “needle in the haystack” approach inherent in electron microscopy did not provide significant particle incidence data when the concentration of the particles was at the borderline of detectability. The fact that most virions are injured or incomplete also adds to the difficulty of correlating particles with other evaluations.

Another explanation for the discrepancy between the presence of RDDP and B particles could be that whole virions do not remain intact very long after they are synthesized and liberated into the milk. The viral cores may, however, be more stable. There is now much evidence for this kind of particle injury. Fig. 1 shows the structural changes in mouse B particles caused by human milk. The extent and severity of damage caused varies from one human milk sample to another. Cream is much more virulcytic than skim milk (16, 36).

Attempts were made to circumvent the loss of particles and RDDP in human milk by either processing it immediately after it was collected or freezing it with Dry Ice as it was taken. Because it was possible that much of the RDDP loss occurred in the milk ducts before the milk was expressed, we made a comparison of “fore” and “hind” milk by having mothers come to the laboratory where the stored fore milk could be taken and processed with the same time delay as the freshly synthesized hind milk from the same donor. The results of RDDP measurements on the fore and hind milk are shown in Chart 1. In 6 of 8 cases, the hind milk had more RDDP than the fore milk. This emphasized the rapidity of viral degradation and helped to explain the erratic RDDP levels found.

Apart from RNase, we still do not know what the destructive factors are nor how to control them. If the variations in RDDP determinations are accepted, the main question at that point was not how much RDDP but with what was it associated? Was it in a MTV-like virion, a leukemia-sarcoma-like virion, or some other kind of particle?

Separation of Type B and C Particles

It had been shown (29) that in a variety of different density
gradients (sucrose, Angio-Conray, potassium tartrate, and potassium citrate) the buoyant density of most of the MuMTV particles was 1.18 g/ml, whereas in CsCl it was greater. Although the type C particles banded less sharply than the MuMTV type B, their buoyant density in the various media, including CsCl, was predominantly 1.16 g/ml. We mixed the 2 types of mouse viruses, RLV and MuMTV, and found that at a density of 1.16 g/ml the distribution of particles was 95% RLV and 5% MTV, whereas at a density of 1.18 g/ml it was 75% MTV and 25% RLV (41). In CsCl the separation was better. The results obtained on human milk fractions taken from a sucrose gradient at the 2 different densities are shown in Chart 2. The particles with which the human RDDP is usually associated have, from the beginning, been found to have the same buoyant density as MuMTV (45). In CsCl gradients most of the human milk RDDP is found at high density (1.18 to 1.22 g/ml) as is MuMTV and not at density 1.15 to 1.17 g/ml as are mouse leukemia virions.

Use of Template Poly(rC)-oligo(dG)

Recently, Smith and Gallo (48) and also McCaffrey et al. (23) found that synthetic poly(rC) template, with oligo(dG)12-18 as a primer, could serve as a specific and sensitive template for tumor viral RDDP. Table 1 gives the RDDP activities found in 25 human milks using this template. The age and parity of the milk donors are included. Forty % of these human milks are considered positive (>150 cpm). There is an increase with parity but the number of tests is not great enough to be statistically significant. Mouse strains which carry MTV of low activity such as RIIIf (tumor incidence 10%) and Af (tumor incidence 47%) develop tumors late in life (15 to 24 months). Neither B particles nor viral antigens are found in their milks until the 3rd to 8th litter; at the 6th lactation there is a correlation between those mice that show MuMTV antigen in their milk and those that eventually

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of the simultaneous method (34). Fore milk is that stored in the breast and expressed first; hind milk is that taken after the fore milk is removed. Only the hind milk contained an appreciable amount of high-molecular-weight (35 S) complex and RDDP activity. The radioactivity at the top of the gradient tube (Fractions 18 to 20) indicates low-molecular-weight complexes.

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develop tumors. The Af subline, which has a higher mammary tumor incidence, shows viral antigens earlier, some of them being positive at the 3rd lactation. American women have an overt breast cancer incidence of only about 6%. Upon extrapolation from the mouse models to women it would be expected that viral expression would be quite late in life and that it might vary greatly from one woman to another as it does from one mouse strain to another.

Cation Preference in RDDP Activity

Use of the template poly(rC)-oligo(dG) provides a means of distinguishing some of the various oncornaviruses by their preference for 1 of the 2 cations, Mg** or Mn** for the RDDP activity (1). The difference in poly(rC)-oligo(dG) directed dGTP-3H incorporation in the presence of the 2 ions for several viruses have been investigated (A. S. Dion, A. B. Vaidya, and G. S. Fout. Poly(rC)-oligo(dG)-directed DNA Synthesis by Particulate Fractions of Human Milk. Submitted for publication to CANCER RESEARCH). Values for MuMTV, MPMV, isolated from a mammary tumor of a rhesus monkey (3, 12), and R35, discovered in a rat mammary tumor (2, 11), are shown in Chart 3, and the ratios of activities with Mg** and Mn** for various oncornaviruses are given in Table 2. The MPMV behaved like the MuMTV while the R35 virus behaved like the mouse, cat, and monkey leukemia-sarcoma viruses. The RDDP activity in human milk particles shows preference for the same cation (Mg**) that is preferred by MuMTV.

Nucleic Acid Hybridization

The relationship of the RNA of MuMTV to RNA found specifically in human mammary tumors has been investigated by means of molecular hybridization experiments (5, 49, 51). Most of the studies indicate some homology between MuMTV RNA and RNA of human breast cancer and human milk. Axel et al. (5) reported that 70 S RNA, associated with RDDP in particles with a buoyant density of 1.16 to 1.19 g/ml from human breast carcinomas, was able to synthesize a DNA that, at least to some extent, complexed with MuMTV RNA. This occurred in 30 of 38 human mammary adenocarcinomas but not in 4 normal breast tissues and 6 fibroadenomas. In other homology experiments (49), a DNA-3H probe was prepared from MuMTV and RLV RNA’s and were tested against monosomal and polysomal RNA from human breast carcinomas. The monosomal RNA did not react. The polysomal RNA hybridized with only the MuMTV DNA-3H and not with RLV DNA-3H.

In still another investigation carried out by Das et al. in Bombay (13), DNA probes, made by using reverse transcriptase and RNA from isolated human milk particles, were reacted with polysomal RNA from human breast tumors. The RNA

Table 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>Type</th>
<th>Host</th>
<th>Mg**/Mn**</th>
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<tr>
<td>R35</td>
<td>C</td>
<td>Rat</td>
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<tr>
<td>MSV</td>
<td>C</td>
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</tr>
<tr>
<td>RLV</td>
<td>C</td>
<td>Mouse</td>
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<tr>
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<td>C</td>
<td>Cat</td>
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<td>C</td>
<td>Gibbon</td>
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<tr>
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<td>Wooly monkey</td>
<td>0.56</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Mouse</td>
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<td>B</td>
<td>Mouse</td>
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<td>A</td>
<td>B</td>
<td>Mouse</td>
<td>18.6</td>
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<td>MPMV</td>
<td>C-B</td>
<td>Rhesus monkey</td>
<td>21.2</td>
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<tr>
<td>Human milk: Pool of 7</td>
<td>Human</td>
<td>5.11</td>
<td></td>
</tr>
</tbody>
</table>

a Ratio of cpm. Values were obtained from Mg** or Mn** optimal concentration or from an average of plateau points.

Chart 3. RDDP activities as a function of concentration (CONC) of Mg** or Mn**. a, MuMTV from strain A mice; b, Mason-Pfizer monkey virus; c, R35 rat virus; Absiccae, all concentrations of Mg** (mM); Mn** (mM X 10).
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from 1 of 3 breast tumors, an undifferentiated carcinoma, showed considerable homology with the DNA probes. Neither mouse embryos, human placenta, nor normal human breast showed any measurable homology with the DNA probes.

Vaidya et al. (51) used single-strand specific nucleases for detecting RNA sequences in human tumors homologous with MPMV and MuMTV DNA probes. Of the 22 tumors tested, none showed homology with MPMV whereas, 8 tumors contained RNA which hybridized from 18 to 77% of the MTV DNA at RNA concentrations of from 2.2 to 5.1 X 10^{-4} moles sec/liter. All 8 of these tumors were carcinomas of the breast; 2 were in situ types, 1 was an infiltrating carcinoma with extensive in situ areas, 1 was precancerous tissue and the other 4 were invasive carcinomas. The number of MuMTV-related RNA molecules/cell based on RNA concentration (moles X sec) half-values of the test RNA's and of MuMTV 70 S RNA was estimated. From 1.5 to 8 MuMTV-related RNA molecules/cell were indicated. Thermal stability of hybrids showed only about 5% mismatching of the base sequences for the molecules of the 2 species. These hybridization results support and help to explain the immunological cross-reactivity observed in other studies (8, 9, 19, 31, 32). Moreover, it appears that only a relatively small segment of nucleic acid may be involved in malignant transformation because the oncogenic activity of MuMTV withstands unusually high doses of ionizing irradiation (4, 17, 27, 28), as does the oncogenicity but not the infectivity of DNA tumor viruses such as SV40 and polyoma (14, 20).

Immunology

Immunology provides still another means for testing the relationship between particles from human milk and MuMTV. A neutralizing effect on MuMTV by human sera was reported in 1971 (9). In this preliminary series, sera from breast cancer patients had a greater neutralizing effect on the mouse virus than control sera. More than 100 sera have now been tested and one-fourth of them showed various degrees of neutralization whether or not the sera came from breast cancer patients. Some sera completely neutralized the RIII mouse virus. Attempts are being made to determine the nature of the neutralizing serum component. It is precipitated as a globulin by ammonium sulfate and is absorbed by RIII milk, but it is not affected by the absorption of the serum with MTV-free C57BL milk or tissue extract. It seems to be a true antibody, but further studies on its nature are in progress.

Immunofluorescent tests have given some indication of a relationship between some human sera and B-particle-producing mouse cells. Priori et al. (34) found that sera of 50% of the breast cancer patients tested, 40% of the relatives of some of these patients, and 15% of all donors reacted with cells of mouse mammary tumor lines that were producing B and C particles. The specificity of these reactions has not yet been determined.

Müller et al. (31) have reported that slices of MuMTV-rich tumors reacted, in immunofluorescence tests, with sera from some women with breast cancer or fibrocystic mastopathy. From the specific fluorescence pattern in the tumor cell, absorption experiments and immunoferritin tests, the human antibody seemed to be directed against an antigen localized mainly on the intracytoplasmic A particles. These results were confirmed later with peroxidase-labeled anti-human IgG. Also Müller et al. (31) have reported that some breast cancer patients seem to have an antigen in their sera that is related to MuMTV antigens.

There are now also evidences for similarity of antigens in MuMTV and in human tumors. Black et al. (7, 8) have utilized the migration inhibition factor test for the demonstration of cellular immune responses to human breast cancer. Positive responses were found in 70% of the tests against autologous in situ breast cancer, 35% of the tests against autologous invasive breast cancer, and 15% of the tests against homologous invasive breast cancer. When leukocytes from unselected breast cancer patients were tested against RIII milk containing MuMTV, positive responses were obtained in 9 (27%) of 34 tests (8). Leukocytes that were responsive to in situ breast cancer tissue cross-reacted with MuMTV-containing RIII milk, but not with MuMTV-free RIII or C57BL milk, in 6 (54%) of 11 tests. Conversely, when leukocytes responsive to MuMTV were tested against homologous in situ breast cancer, positive responses were found in 36 (55%) of 66 tests. Leukocytes that failed to respond to MuMTV rarely (2 of 61) responded to homologous in situ breast cancer tissues. It appears that an appreciable proportion of human in situ breast cancers contain an antigenic component similar to that of MuMTV.

Still another indication of mouse and human virus similarities has been reported by Hoshino and Dmochowski (19). Using peroxidase-coupled anti-human globulin it was shown that sera from human breast cancer patients formed a virus-specific precipitate in thin sections of mouse mammary tumor examined with the electron microscope. This was interpreted as a specific antigen-antibody reaction between mouse virus and antibody to human virus arising in a human subject.

Other Candidate Mammary Tumor Viruses

Over the years the mouse has served as a model to bring us to our present state of knowledge concerning a viral etiology of breast cancer in humans. We now have candidate viruses in 2 other species, rhesus monkey (3, 12) and rat (2, 11). Of these, the properties of the candidate human virus more closely parallel those of the mouse than do the other two; RNA from the monkey (MPMV) virus does not show any homology to probes made from MuMTV. In addition to the properties described above, neither the mouse virus nor the human candidate virus have adapted easily to tissue culture infection and replication; both MPMV and R35 have adapted readily.

Explants of mouse mammary tumors can, however, be established as cell lines that will continually synthesize MuMTV; but explanted human tumors have not, in spite of much effort, been shown to synthesize any mammary tumor virus. Herein lies the main difference between the manifestation of a viral etiology of breast cancer in the 2 species; the mouse is an excellent virus producer, and man is a
comparatively poor virus producer. From all of the accumulated evidence, however, this need not detract from the notion of the viral etiology of breast cancer in man. The biology of the 2 species, particularly the hormonal and reproductive characteristics on which the mammary tumor virus is so dependent, are very different. We pointed out earlier that many mice and mouse cells seem to carry an unexpressed MuMTV genome (25). The evidence now available indicates that a mammary tumor viral genome is present in all mice (52) and that it can usually be derepressed by chemicals, irradiation, etc. (6, 43). To date, however, there is no evidence that a MTV genome is present in all humans. In fact, no DNA homologous with MuMTV nucleic acid can be found in humans (53). It is possible, however, that a breast cancer virus is widespread in humans (39) although its full expression may be relatively rare. Its partial expression, in the production of antigens, RDDP, or cell transformation may be more common. Permissive cells and proper conditions may allow in vitro demonstration of putative human mammary tumor virus infection.

Despite the fact that the route of infection (whether via milk, gametes, or other means) in humans is unknown, an RNA tumor virus with properties similar to those of MuMTV seems to have left definite traces in some women.

REFERENCES


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Fig. 1. Effect of human milk on MuMTV virions. Control virions incubated in phosphate-buffered saline (a) and virions incubated in human milk (b, c, and d) at 37° for 18 hr. The control virions (a) were well preserved, and showed head and tail form and surfaces covered with spikes (S). The human milk caused various kinds of degradation: injury to spikes (b), removal of spikes (nt), and of cores (c), and complete degradation of particles (d), × 95,000.
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