Karyotypic, Virologic, and Immunologic Analyses of Two Continuous Lymphocyte Lines Established from New Zealand Black Mice: Possible Relationship of Chromosomal Mosaicism to Autoimmunity

abbreviation: NZB, New Zealand

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ABSTRACT Two continuous-suspension lymphocyte lines were isolated from the spleen and fibrosarcoma of a New Zealand Black female mouse. A C-type virus with a density of 1.16 g × cm⁻³, 70S RNA, and RNA-directed DNA polymerase activity was isolated. The virus was infectious for NRK, NZB, (NZB x NZW)F₁, and (NZW x NZW)F₁, embryos, and for BALB/c 3T3 cells, but not for NIH Swiss cells. All cells from established lymphocyte cultures, as well as some embryo cells from New Zealand Black mice, showed karyotypic abnormalities. The possibility of chromosomal mosaicism is suggested.

Human systemic lupus erythematosus and the related diseases in New Zealand black (NZB) mice and in (NZB x NZW)F₁ hybrid mice are immunologic disorders with a plethora of symptoms, often leading to death (1). These diseases are associated with the synthesis of various autoantibodies against erythrocytes and both nuclear and nucleolar constituents. In addition, the NZB mice make antibodies to Gross-like leukemia virus. Some examples of the specificities of the autoantibodies are: antibodies against native double-helical DNA, DNA-histone complexes, single-stranded DNA, and nucleolar RNA. Often, the most serious organ involvement in these diseases has been shown to be caused by phlogogenic antigen-antibody complexes (2-4) in which at least some of the above mentioned antigens are present.

Aside from the interesting and well-studied issue of the pathogenesis of organ injury in patients with systemic lupus erythematosus and in NZB mice, is the question of why these patients and mice make autoantibodies to erythrocytes and to various nuclear materials. Some of the possibilities are that hosts (a) are not able to repair nucleic acids properly, and altered nucleic acids might be released from cells into circulation. Such altered nucleic acids might have an abnormal catabolic fate and/or antigenicity. This situation would be analogous to the defect in the repair of DNA that occurs in the human disease, xeroderma pigmentosum (5); (b) are deficient in or have abnormal nucleases and perhaps other catabolic enzymes that result in the formation of immunogenic breakdown products from spent, autologous cells. (c) have a defect in some intracellular structure such as the nuclear and/or plasma membrane that results in loss of ability to keep nucleic acids in the proper configuration or intracellular compartment. (d) harbor or are infected by a virus, the genome of which is immunogenic because of a different configuration or nucleotide composition than that of the host, and thus tolerance to autologous nucleic acids is terminated; this would be similar to the experimental termination of tolerance to thyroglobulin in subjects exposed to chemically altered native thyroglobulin (6). Also, viral infection could lead to chromosomal rearrangement with consequent changes in important control functions of the host genome.

In short, it seems possible that cells from patients with systemic lupus erythematosus or from diseased NZB mice are variants in which one or a combination of the defects listed above has occurred. Detection of such a variant(s) could not only be important for determination of the pathogenesis of systemic lupus erythematosus, but also, as with other variant cells, might be helpful in elucidation of the nature, repair, or catabolism of nucleic acids in normal cells. Here we report briefly the establishment and some of the properties of two different continuous lymphocyte lines from the spleen and a fibrosarcoma of an NZB mouse. Interesting karyotypic abnormalities and a unique “C-type” virus were noted in both cell lines.

MATERIALS AND METHODS

Establishment and Maintenance of the Cell Lines. Two lymphoid cell lines were established from a 1-year-old NZB female mouse that had spontaneously developed a fibrosarcoma. One line was established from fragments of a fibrosarcoma with a considerable lymphoid infiltrate and the other from splenic fragments (7). Both cell lines were maintained in suspension on a gyratory shaker (8, 9). According to our convention, we designated the line established from the tumor SCRF 60A, and the line established from the spleen SCRF 61B.

Virologic Studies. Cultures of SCRF 60A cells were initiated at a density of 8.0 × 10⁴ cells per ml and grown for two cellular generations in complete medium containing 0.05 μCi of [²⁻¹⁴C]uridine/ml (50 Ci/mol). Cells were removed from the culture fluid by centrifugation at 800 × g for 10 min. The cell-free media were clarified by centrifugation at 13,200 × g (average) for 10 min. The virus from 250 ml of clarified tissue-culture fluid was concentrated by isopycnic banding at 96,300 × g (average) through 6 ml of sucrose with a density of 1.0583 g × cm⁻³ onto a 3-ml cushion of sucrose with a density of 1.1867 g × cm⁻³. The turbid band at the interface of the two sucrose solutions was collected and diluted 3-fold with 10 mM Tris-HCl (pH 7.4) that contained 100 mM NaCl and 1 mM EDTA. The diluted band was sedimented to its equilibrium density for 4.5 hr at 96,300 × g (average) through a linear su-
FIG. 1. Electron microscopy of infected cells. Cells from line SCRF 60A were fixed (see Methods) and examined by electron microscopy. The lymphoid morphology of the cells and the presence of a large number of virions are shown. In B, (arrow) a typical budding "C-type" particle is shown and in D, (arrow) the double-membrane structure of the virus is shown.

cross gradient in Tris–NaCl–EDTA between the densities of 1.0583 g cm⁻³ and 1.1867 g cm⁻³. Fractions were collected through a Gilford recording spectrophotometer (0.5-cm light path), and the χCH₂COOH-precipitable radioactivity was determined as described in ref. 10.

Fractions that contained radioactivity were pooled and diluted 4-fold in Tris–NaCl–EDTA; particles were "pelleted" by velocity sedimentation at 308,000 × g (average) for 4 hr. The pellets were suspended in the same buffer, and RNA was isolated by treatment with 1% sodium dodecyl sulfate (w/w) and 1 mg of nuclease-free Pronase (Calbiochem)/ml for 1 hr at 37°. After isolation, the RNA was heated at 60° for 30 min. The sedimentation velocity of the RNA was determined by sedimentation through 16 ml of linear sucrose–sodium dodecyl sulfate gradients at 79,500 × g (average); fractions were collected and radioactivity was determined as described in ref. 11. Unlabeled 18 and 28 S ribosomal RNAs were isolated from diploid lymphocytes (11) and were used as a marker.

The viral infectivity of normal rat kidney (NRK), BALB/c 3T3, and NIH Swiss mouse-embryo cells, and cells from embryo cultures of NZB, NZW, and (NZB x NZW)F₁ hybrid was studied by the XC test of Klement et al. (12). The ability of the virus to induce synthesis of RNA-directed DNA polymerase (reverse transcriptase) with oligo (dT) · (rA)₉ as a template was kindly tested (13) by Dr. Raymond Gilden.

Immunologic Studies. The surfaces of viable cells were studied during the period of logarithmic growth for the presence of immunoglobulin, theta antigen (θ), and Gross soluble antigen by an immunofluorescent assay (14). Antibody to θ and Gross soluble antigens was kindly provided by Dr. Michael B. A. Oldstone.

To test for synthesis of antinuclear antibody by these cells, the globulin fractions from 130 ml of complete medium in which cells had grown to a density of 2.0 × 10⁹–3.0 × 10⁹ was precipitated at 4° with ammonium sulfate at a final concentration of 50% (w/w), pH 7.4. Precipitated globulin was suspended in 4 ml of phosphate-buffered saline (0.15 M NaCl–10 mM PO₄³⁻, pH 7.2) and dialyzed against the same buffer to remove remaining ammonium sulfate. The globulin fraction was tested for the presence of antinuclear antibody by immunofluorescent assay (15).

To determine if any of the cells were synthesizing antibodies to erythrocytes, we collected 1.0 × 10⁶ cells from a culture in the logarithmic phase of growth and tested them by a modification of the Jerne plaque assay that has recently developed for the detection of spleen cells that synthesize autoantibodies to erythrocytes (16).

Electron Microscopy. Cells were fixed, stained, and examined as described in ref 17.

Karyotypic Analysis. For chromosome preparations, exponentially growing cells from the established lines SCRF 60A and SCRF 61B were incubated for 2 hr in 0.1 μM Colcemid (GIBCO) before harvesting. Fibroblast cultures were prepared from pooled and single embryos of NZB x NZB, NZW x NZB, and NZW x NZW matings. After 2–4 days, the primary cultures were exposed to 0.1 μM Colcemid for 4 hr and then harvested. Both types of cells were treated with a hypotonic solution of 75 mM KCl for 12 min and fixed in 3:1 methanol–glacial acetic acid. Slides were prepared by the air-drying technique.

For demonstration of Giemsa banding patterns on chromosomes, the well-dried slides were dipped into 0.25% trypsin solution (DIFCO) for 30 sec, rinsed in isotonic saline, and stained in Giemsa (GURR) solution (pH 6.8) for 5–15 min by a modification of the method described by Seabright (18).
Staining with quinacrine mustard and fluorescent microscopy were done as described in ref. 19. Both staining techniques were applied to the cell lines. Karyotypes were prepared according to the recommendations by the Committee on Standardized Genetic Nomenclature for Mice (20).

RESULTS

General properties of the cell line

After 3 weeks, continuous-suspension cell lines were established from the spleen and fibrosarcoma of the female NZB mouse. The cells grew in suspension to a maximum density of about $3.5 \times 10^6$ cells per ml (generation time 18 hr).

By light and electron microscopy, the cells resembled relatively undifferentiated lymphoblasts (Fig. 1). The cells had a high nuclear to cytoplasmic ratio, few membrane-bound polyribosomes, and almost no lysosomal-type structures. With these features, the cells resembled the majority of diploid lymphocyte lines in continuous culture in our laboratory (21).

Virologic properties of the cell line

As can be seen from electron micrographs (Fig. 1b,c,d), the NZB cells (SCRF 60A) in continuous culture produce a large number of budding viral particles with the classic double membrane and a "C-type" morphology (arrows) of RNA tumor viruses. The intact virus particles had a bouyant density in sucrose of 1.16 g X cm$^{-3}$ (Fig. 2a), and the isolated, heated (60° for 30 min) viral genome had sedimentation velocities of 35 S and 4 S (Fig. 2b). Release of the 4 S RNA molecule after conversion of the high molecular-weight form (70 S) to the 35 S form was reported in ref. 22.

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Fig. 2. A. Isopycnic sedimentation of virus isolated from SCRF 60A cells. Cells and virus were labeled with [5-14C]uridine (50 Ci/mol) sedimented to equilibrium (see Methods). The intact virus had a density of 1.16 g X cm$^{-3}$. O—O = $\rho$; A$^{35S}$ nm; O—O = DPM [5-14C]uridine. B. Velocity sedimentation of viral RNA. Viral RNA was purified and sedimented through 16 ml of linear sucrose-sodium dodecyl sulfate gradients (see Methods). Unlabeled 18 and 28 S ribosomal RNAs were used as markers. A$^{28S}$ nm; O—O = DPM [5-14C]uridine.

Fig. 3. Antigenic properties of the cell surface of SCRF 60A and SCRF 61B cells. Cells were studied for the presence on the surface of $\theta$, IgG, and Gross soluble antigens (see Methods). $\theta$ antigen is shown on the surface of SCRF 61B cells (a) and Gross soluble antigen is shown on the surface of SCRF 61B cells (c). As a control, the positive staining for the surface of IgG on surfaces of spleen cells from an NZB female mouse is shown in b.

Fig. 4. Karyotypic analysis of the cell line SCRF 60A. Exponentially growing cells from line SCRF 60A were arrested in metaphase; metaphase spreads were prepared and studied by the Giemsa banding method (see Methods). The marker (M) chromosome and the deletion of the distal X(Xp) are evident.
By the 4th stained with quinacrine mustard, 2968 Immunology: not but Gross from that the virus antigen 0 was additional synthesis was Immunoglobulins associated with immunofluorescent assay satisfactory photography peripher. The on tected RNA-directed immunofluorescent and thymus for controls" antigen was faintly virus, for the 50% present staining of the virus isolated from SCRF cells differs in its tropism from Gross virus, the present results suggest that this is the case.

Immunologic properties of the cell line

θ antigen was present on the surfaces of all SCRF 61B cells, but was not detected on surfaces of SCRF 60A cells (Fig. 3a). Immunoglobulins associated with cell surfaces were not detected on the surfaces of SCRF 61B cells, but with optimal immunofluorescent methodology about 50% of the SCRF 60A cells had faintly positive fluorescence, which was granular and peripheral. The intensity of this fluorescence was so low that satisfactory photography was impossible. To ensure that the immunofluorescent assay was working properly, "positive controls" for detection of θ antigen and immunoglobulins associated with cell surfaces were included in each study; thymus and spleen cells were used, respectively, from 6-month-old NZB female mice. The positive staining of the spleen cells for immunoglobulins associated with cell surfaces is shown in Fig. 3b. Positive staining of thymus cells for θ antigen was also observed. The surfaces of all cells of both lines showed strong positive staining for Gross soluble antigen by our immunofluorescent assay (Fig. 3c). No antinuclear antibody or antibody against erythrocytes could be detected in the concentrated tissue culture fluids from these cells by immunofluorescence or by the modification of the Jerne plaque assay (see Methods).

Karyotypic analyses of the cell lines and embryo fibroblasts*

Cell Lines. 41 chromosomes were found in 71 of 80 metaphases counted. 25 Karyotypes were prepared from each line; all of them included an extra telocentric chromosome (M) of the size of a no. 14 chromosome, that exhibited a band pattern different from that in normal mouse chromosomes (20). The marker chromosome (M) (Fig. 4) is characterized by two distinct, darkly Giemsa-stained bands at one-third and two-thirds of the total length, the distal one being darker. In addition, a deletion involving one of the X chromosomes (X0 in Fig. 4) was present in all the karyotypes from both lines. X0 appeared to be missing part of the faintly staining distal band.

* 25 Additional continuous suspension cultures were established from different New Zealand mice and are being studied. All produce virus and in eight where cytogenetic analysis has been completed, 41 chromosomes, including the marker, and the deleted X chromosome were found.
Embryo Fibroblasts. Chromosome counts and karyotypic analysis of the embryo cells revealed 8 of 38 metaphases from pooled NZB x NZB embryos (Fig. 5) and 5 of 28 cells from pooled NZB x NZW embryos with 41 chromosomes due to the presence of a marker chromosome similar to the one found in the lymphoblastoid lines. The deleted X chromosome (X<sup>D</sup>) was not found in any of the embryo fibroblasts. Pooled NZW x NZW and NZW x NZB fetuses, as well as two single NZB x NZW and NZW x NZB fetuses, had normal chromosome counts and karyotypes.

Increased incidence of chromosome breakage was observed, but no attempts were made to quantify this finding.

CONCLUSIONS

We have isolated from the spleen and fibrosarcoma of a female NZB mouse two separate continuous cultures of lymphoid cells. These cells do not produce antinuclear or anti-erythrocyte antibodies but synthesize a great deal of virus with the morphologic and biochemical properties of RNA tumor viruses. The fact that these cells fail to synthesize detectable amounts of antibody to erythrocytes or nucleic acids is not surprising. Most cell lines originate from very few clones, and without use of a selective pressure the probability of obtaining cells that synthesize antibody to any given antigen is very low.

Previous studies of the chromosomes of NZB mice and NZB x NZW hybrid mice, without use of banding techniques, either detected no morphological or numerical abnormalities (23, 24) or described significantly higher incidences of chromosome breaks in NZB mice compared to other inbred mouse strains (25).

The implication of the chromosomal abnormalities, the extra marker (M), and the deletion of the distal X (X<sup>D</sup>), which we found in the NZB cell lines, for virus production and the autoimmune process remains to be investigated. The increased tendency of chromosome breakage, however, could well be related to the presence of virus in these cells. Chromosomal rearrangements are likely to occur as results of increased breakage.

Since X<sup>D</sup> was found in both cell lines, it probably did not originate in culture, but rather was present in the mouse from which both lines were derived. The piece missing from X<sup>D</sup> does not appear to be translocated onto another chromosome; M, however, may contain X chromosomal material.

A study of the association of chromosomal abnormalities and disease in the NZB or (NZB x NZW)<sub>F<sub>1</sub></sub> hybrid mice is more difficult than the usual genetic studies, since an immune response is clonal and is, in this sense, similar to granulocytic leukemia or multiple myeloma. Thus, karyotypic abnormalities might only be expected in cells directly involved in the autoimmune disease process, and this would be similar to the observation that the “Philadelphia” chromosome is found only in hematopoietic cells of patients with granulocytic leukemia (26). Karyotypic analysis of patients with systemic lupus erythematosus, NZB, (NZB x NZW)<sub>F<sub>1</sub></sub> hybrid mice, and NZW mice will be reported elsewhere. Nevertheless, these cell lines should be useful for determining if any biochemical abnormality is present in the cells of the NZB mouse.

The results presented here differ from those reported by Levy and Pincus (27) in two important aspects. They prepared a pseudotype sarcoma virus that presumably carries NZB virus structural polypeptides, [M-MSV(NZB)]. The pseudotype virus was only active on NRK cells. In the absence of quantitative estimates of viral infectivity on the different cell lines, it is not possible to completely specify host cell restriction. It seems likely that our preparation contains more infectious units than those reported by Levy and Pincus. This suggestion is supported by the fact that they (27) were unable to propagate the virus in vitro.

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