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Immunology

Novel B-cell maturation factor from spontaneously autoimmune viable motheaten mice

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ABSTRACT **Both *in vivo* and *in vitro*, mice homozygous for the viable motheaten mutation show severe immunodeficiency, polyclonal B-cell activation and Ig secretion, and spontaneous production of a lymphokine [B-cell maturation factor (BMF)] that directly drives the maturation of normal or tumor B cells to the state of active Ig secretion. BMF from motheaten mice is distinct from previously identified forms in its cells of origin (B cells) and biochemical characteristics (apparent M_r 15,000 by gel filtration and NaDodSO$_4$/PAGE; pI 4.3 by chromatofocusing). Among the known murine single-gene models of autoimmunity, only motheaten mice show high levels of spontaneous BMF production, which therefore may be an important component in the development of this form of autoimmunity/immunodeficiency disease. The coincidence of spontaneous BMF production and uncontrolled Ig secretion within the same mutant mouse constitutes the strongest evidence to date for a significant physiological (*in vivo*) role for BMFs.**

Two common features of autoimmune disease are polyclonal B-cell activation and Ig (including autoantibody) production (1, 2). In recent years, many laboratories have investigated soluble molecules (lymphokines) that affect B-cell function (3, 4). Molecules promoting B-cell replication or differentiation have been reported, although the molecular characteristics and full range of biological activities of these molecules have not been clarified completely.

Mice homozygous for the allelic, recessive single-gene mutations called motheaten (5, 6) and viable motheaten (7) (gene symbols *me* and *me^v*, respectively) show the most severe genetically determined autoimmune syndromes known in mice. Animals homozygous for either allele show signs of autoimmune disease within the first week of life, develop strikingly increased serum IgM levels by age 3 weeks, and have mean lifespans of 3 and 9 weeks, respectively. We have recently concentrated on investigations of the *me^v* allele, since mice homozygous for this allele reach adulthood and live for many weeks thereafter, whereas mice homozygous for the *me* allele generally die shortly after weaning.

In this report, we show that sera and tissue culture supernatants of cells from lymphoid tissues of *me^v* mice contain high levels of a lymphokine active as a B-cell maturation factor (BMF). Molecules with this activity are able to directly drive normal resting B lymphocytes (8, 9) and certain tumor B cells (9) to active Ig secretion and have been shown to affect B cells isolated from all lymphoid organs of all strains of mice of all ages tested (8). BMF derived from *me^v* mice is distinct from previously described species in its biochemical characteristics, functional properties, and cells of origin. The spontaneous *in vivo* production of high levels of this BMF may be the proximal cause for the polyclonal Ig production, humoral autoimmunity, and immunodeficiency that are characteristic of *me* and *me^v* mutant mice. Other murine single-gene models of autoimmunity do not appear to produce high levels of BMF and show overall patterns of immunological dysfunction that are distinct from that seen in motheaten mice.

MATERIALS AND METHODS

Mice. The *me^v* mutation arose in the C57BL/6J strain and is maintained by mating heterozygotes (7). Control mice were either nonmotheaten littermates (*me^v*/+ or +/+) or normal C57BL/6J animals. The *gld* (generalized lymphoproliferative disease) mutation arose and has been maintained in the C3H/HeJ strain (10). The *lpr* (lymphoproliferation) mutation was transferred to the C3H/HeJ strain from the MRL/Mp strain (11). For both the *lpr* and *gld* mutations, homozygous mutant animals were tested against their co-isogeneic or congeneic wild-type (+/+) inbred partners. Mice of the BXSB/Mp strain carry an autoimmune acceleration factor (*Yaa*) on their Y chromosome originally derived from strain SB/Le (12). The Y chromosome of C57BL/6J was transferred to the BXSB/Mp strain by multiple (more than eight) backcrosses. Male BXSB/Mp Y^{SB} (autoimmune) mice were compared to their Y-consomic (differing only at the Y chromosome) partners BXSB/Mp Y^{B6} (nonautoimmune) in this study.

Cells. Mice were killed by CO_2 asphyxiation, after which their spleens were removed, dissociated into Earle's balanced salt solution, and depleted of erythrocytes by treatment with hemolytic Gey's solution as described (13). Resting spleen cells were isolated by Percoll density gradients at a density $\geq$ 1.085 g/ml. Cells isolated from mice were always cultured (including the generation of supernatants) in serum-free Iscove's modified Dulbecco's medium supplemented with albumin, transferrin, and soybean lipids (IMDM-ATL) as described (14). WEHI-279.1 tumor B cells (15, 16) were grown and cultured in a fetal bovine serum-containing RPMI-1640-based culture medium as described (8). Erythrocyte-depleted *me^v* spleen cells were stained for sorting by using fluorescein isothiocyanate-conjugated F(ab')$_2$ fragments of goat anti-mouse Ig antibodies (Cappel Laboratories). Sorting was done with an Ortho 50-H sorting cytofluorograph.

Cultures and Other Assays. BMF-containing supernatants were generated by culturing whole or sorter-separated spleen cell populations in IMDM-ATL for 2–3 days, followed by sterile filtration and storage at 4°C or −20°C (with no apparent difference in stability). BMF test cultures were set up in 0.2-ml flat-bottom microculture wells, with 200–1000 responding cells per well. After 3 days, the cultures were analyzed by the polyclonal protein A anti-IgM reverse plaque assay (17). Amounts of BMF in a test sample were calculated by comparison to a standard curve generated with *me^v* spleen cell supernatant and WEHI-279 cells, with one

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Abbreviations: BMF, B-cell maturation factor; IMDM-ATL, Iscove's modified Dulbecco's medium supplemented with albumin, transferrin, and lipids; PFC, plaque-forming cell(s).

unit of activity defined as that amount of BMF giving 50% maximal plaque-forming cell (PFC) response in a standard culture (18). IgM levels were determined by an enzyme-linked immunosorbent assay (19) using monoclonal anti-μ heavy chain antibodies bound to the plate and alkaline phosphatase-coupled monoclonal anti-κ light chain antibodies as the developing reagent.

Biochemical Analyses. Gel filtration chromatography was done with a 2.5 × 100 cm column of Sephadex G-75 in 1 M NaCl/10 mM Hepes, pH 7.2. The sample was applied in 5 ml, and 10-ml fractions were collected, dialyzed three times vs. phosphate-buffered saline (pH 6.8; P_i/NaCl) and once vs. IMDM-ATL, and filtered under sterile conditions. Chromatofocusing was done with a 1 × 45 cm column of Polybuffer exchanger PBE 94 (Pharmacia), with 2 ml of Sephadex G-25 layered on top for even sample application. The column was equilibrated with 0.025 M histidine·HCl (pH 6.2), and the sample was dialyzed vs. the same buffer, applied to the column, and eluted with Polybuffer 74/HCl, pH 3.0. Fractions of 10 ml were collected, dialyzed, filtered, and tested as described above. $NaDodSO_4$/PAGE was performed after concentrating the sample by CX-10 (Millipore) ultrafiltration, adding the reducing $NaDodSO_4$ sample buffer, and reconcentrating. Samples were then run on 15% gels as described by Laemmli (20). The gels were sliced into 1-mm pieces, which were finely ground by using a microgrinder and eluted (for 2 days with vigorous shaking) into 1 ml of P_i/NaCl containing 1% bovine serum albumin. After the gel fragments were removed by centrifugation, the samples were dialyzed once vs. 8 M urea, three times vs. P_i/NaCl, and once vs. IMDM-ATL, filtered under sterile conditions, and tested.

RESULTS

Supernatants prepared by culturing *me*^{v} splenocytes for 2 days in serum-free medium are able to induce active Ig secretion, as measured by the polyclonal IgM reverse plaque assay, from both normal resting B lymphocytes and inducible WEHI-279 tumor B cells. Fig. 1 shows dose–response curves of the PFC obtained from both of these B-cell populations cultured with different amounts of *me*^{v} supernatant. The maximum activity and titer of such supernatants are comparable to those of supernatants derived from T cells (9). Fig. 1 also shows the basis for comparing different samples by calculating standard units of activity. The amount of material necessary to give 50% of the maximal response seen in such a supernatant titration, using WEHI-279 as the indicator cells, is defined as one unit of activity, and the titers of test samples are determined by comparison to such a standard curve (18).

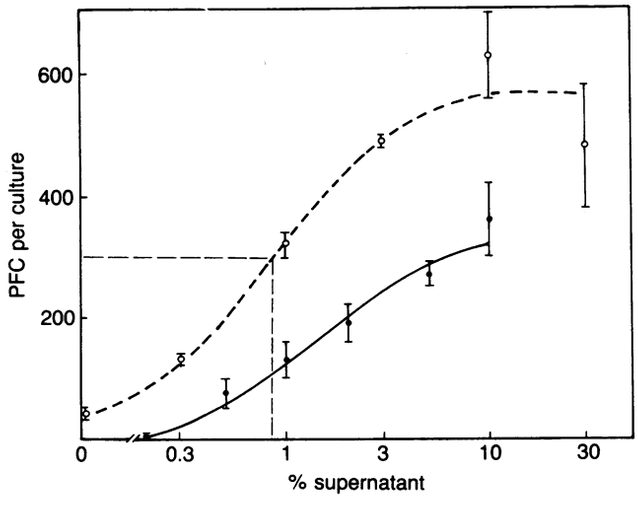

FIG. 1. Dose–response curves of normal resting spleen cells (●——●) and WEHI-279 tumor B cells (○---○) responding to supernatant from *me*^{v} spleen cells. The 50% maximal PFC response of WEHI-279 cells, which defines one unit of BMF activity, is also shown.

Fig. 2 shows the ontogeny of IgM and BMF levels in sera of normal and *me*^{v} mice as well as the titers of BMF generated *in vitro* in spleen cell cultures from these mice. Although the levels of serum IgM are 2- to 3-fold higher in *me*^{v} than in control mice before age 2.5 weeks, after this time *me*^{v} mice show 10- to 20-fold higher serum IgM levels than do control littermates. In contrast, the levels of BMF measured in *me*^{v} sera or spleen cell tissue culture supernatants are essentially constant throughout life. Normal control mice do not show BMF activity from either source. Sera from viable motheaten mice generally show >100-fold more BMF activity per unit volume than do the most stimulatory *in vitro* culture supernatants. Comparable supernatants also can be generated by culturing cells from other *me*^{v} lymphoid organs (lymph node, bone marrow, and thymus; data not shown). The release of BMF activity into culture supernatants is rapid, beginning within minutes of establishing the cultures (data not shown).

The cellular origin of *me*^{v} BMF was investigated by culturing different spleen cell populations isolated by cell sorting and then assaying the resulting supernatants for BMF activity (Fig. 3). Viable motheaten spleen cells were sorted into the three populations shown in Fig. 3*A* by the combination of

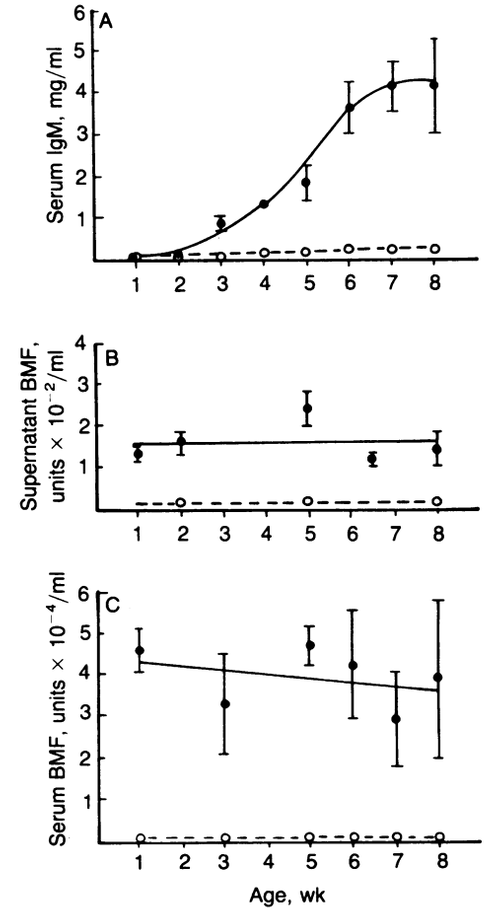

FIG. 2. Ontogeny of *me*^{v} (●——●) and control (○---○) serum IgM (*A*), BMF in spleen cell tissue culture supernatants (*B*), and serum BMF (*C*). Error bars are not shown for the control data since they are <10%.

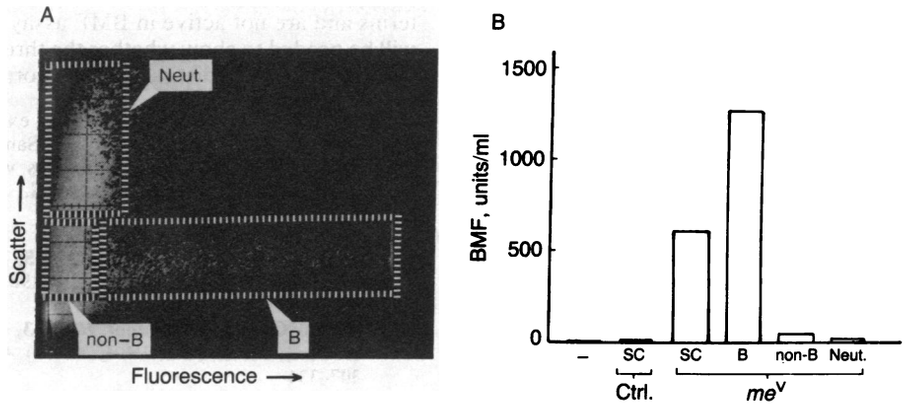

FIG. 3. Source of *me*^{v} BMF. (*A*) Cell populations isolated from *me*^{v} spleen cells using a cell sorter. (*B*) BMF levels derived from the various cell populations. Data from a representative experiment are shown. –, Medium alone. Ctrl., control: SC, unseparated spleen cells. *me*^{v}: SC, unseparated spleen cells; B, Ig(+) spleen cells; non-B, small Ig(–) spleen cells; Neut., large Ig(–) spleen cells (neutrophils).

scatter and fluorescein isothiocyanate-conjugated anti-Ig immunofluorescence. All of the BMF activity from *me*^{v} spleen cell populations derived from the Ig(+) B-lymphocyte pool; essentially none came from the Ig(–) non-B-lymphocyte or large Ig(–) [mostly neutrophil (7)] cell populations (Fig. 3*B*).

Several of the molecular properties of *me*^{v}-derived BMF were determined by using standard biochemical fractionation techniques, followed by titration of the various fractions for units of BMF activity (Fig. 4). In gel filtration chromatography with Sephadex G-75, *me*^{v} BMF was eluted as a single sharply-defined peak of approximate M_r 15,000 (Fig. 4*A*). In column chromatofocusing, a single pI of 4.3 was evident (Fig. 4*B*). In NaDodSO$_4$/PAGE, a major peak at M_r 15,000 was seen, with minor peaks at M_rs 22,000 and 38,000 (Fig. 4*C*).

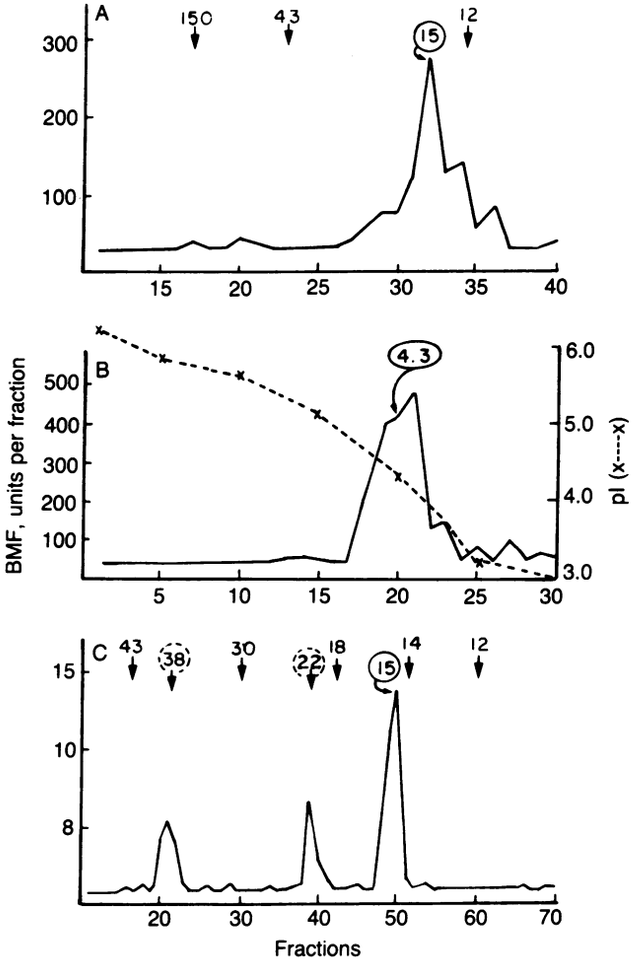

FIG. 4. Biochemical properties of *me*^{v} BMF. Fractionation of BMF activity from *me*^{v} spleen cell supernatant on Sephadex G-75 gel filtration (*A*), column chromatofocusing (*B*), and NaDodSO$_4$/PAGE (*C*). In *A* and *C*, M_rs of markers and activity peaks are noted in $M_r \times 10^{-3}$.

Finally, four single-genetic-factor mouse models of autoimmunity were compared for their production of BMF by spleen cells *in vitro*. Viable motheaten (*me*^{v}), lymphoproliferation (*lpr*), generalized lymphoproliferative disease (*gld*), and BXSB male mice, plus their appropriate controls, were tested at their respective ages of active autoimmune disease (7, 10–12). Although all of these models show severe polyclonal Ig production and autoimmunity and some BMF activity in spleen cell culture supernatants, *me*^{v} mice produced >10 times the amount of BMF elaborated by cells of the other three autoimmune strains of mice (Fig. 5). In other experiments (data not shown), *me*^{v} mice as old as 6 months and the other autoimmune strains as young as 1 month were tested, with no change in the patterns shown in Fig. 5.

DISCUSSION

If lymphokines play a major role in regulating B-lymphocyte function, disorders in the production of lymphokines or in B-cell responses to them could represent important elements in the etiology of immunological disease. We have identified such a case of abnormal lymphokine production, and the data are presented here. Viable motheaten mice show severe autoimmunity, polyclonal Ig production, and immunodeficiency and die by several months of age (7). They also spontaneously produce high levels of a lymphokine (BMF) that

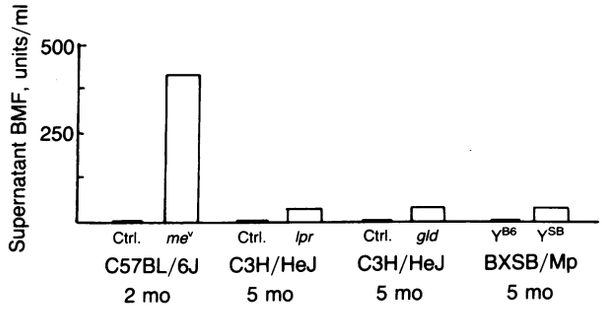

FIG. 5. Comparison of autoimmune mouse strains for their production of BMF in spleen cell supernatants. Data from representative pairs of mice are shown.

has the property of directly driving resting B lymphocytes to active Ig secretion. This production of BMF may directly cause the polyclonal Ig production and humoral autoimmunity seen in these mice. Also, the lack of resting B cells able to respond specifically to a discrete antigenic challenge (unpublished data) may be due to the fact that all me^v B cells have been driven already to active Ig secretion. Mice homozygous for the original *me* allele resemble those with the me^v allele documented here in terms of their production of a similar BMF (data not shown). Sex and background strain (C57BL/6J vs. C3Heb/FeJ) also seem to be without effect on BMF production and Ig levels (data not shown).

The me^v model is distinct from the other single-gene models of murine autoimmunity in terms of its profound immunodeficiency, short lifespan, specific aspects of immunopathology, and the high levels of spontaneous BMF production that are documented here (7, 10–12; unpublished data). Thus, production of BMF may be a characteristic and important part of the mechanism leading to a unique type of immunological disease. Other authors have concluded that MRL-*lpr* mice have high levels of B-cell growth and differentiation factors, based on assays of B-cell proliferation and antibody secretion (21, 22). However, detailed functional and biochemical studies on the molecules active in that system are lacking, and both of these assays might reflect the activity of B-cell replication factor(s) (23, 24). As shown here, mice homozygous for *lpr* do not show the high levels of BMF seen in me^v animals. It is possible, therefore, that the *lpr* and me^v mutations lead to overproduction of B-cell replication and maturation factors, respectively, and thus are models for two distinct types of autoimmune disease.

The presence of high serum levels of BMF in me^v mice is quite striking and suggests either rapid production or substantial stability of BMF *in vivo*. The mechanism leading to the production of BMF in me^v mice is as yet unknown. However, as shown here, it is in full operation within the first week of life, in contrast to normal immune competence, which develops fully only after weaning.

This report documents a novel species of BMF. In contrast to two other BMF molecules (S26.5 and γ interferon), which are derived from T lymphocytes (9, 25), me^v BMF is produced by B cells. Furthermore, me^v BMF is >1 unit more acidic (pI 4.3) and much smaller on gel filtration (apparent M_r 15,000) than the previously described species (pI 5.5–6 and apparent M_r 50,000–55,000) (18). Neither S26.5 nor me^v BMF show the antiviral activity (18, 25), binding and inhibition by anti-γ-interferon antibodies (25), or the B-cell toxicity (to be reported elsewhere) displayed by γ interferon. Nevertheless, all three BMF molecules share the property of inducing normal resting B cells and some tumor B-cell populations to begin active Ig secretion. The other recognized lymphokines interleukin 1, interleukin 2, interleukin 3/burst-promoting activity, and granulocyte/macrophage-colony-stimulating activity are distinct entities in molecular terms and are not active in BMF assays (18). Future studies will be needed to show whether the three different BMF species play similar or distinct roles in normal immune function.

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