

Papers

Intracellular staining of Mx proteins in cells from peripheral blood, bone marrow and skin

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Abstract

Aim/background—The Mx proteins are known to be specifically and dose dependently induced in mononuclear cells (MNC) by type I interferons (IFN). The aim of this study was to establish a staining method for the human intracellular Mx proteins, MxA and MxB, in leucocytes and bone marrow and skin cells.

Methods—Several monoclonal antibodies directed against the MxA and MxB proteins were generated. These antibodies were used to stain Mx proteins in both frozen and paraffin wax sections using the standard alkaline phosphatase anti-alkaline phosphatase (APAAP) method.

Results—Granulocytes, monocytes and lymphocytes extracted from freshly collected blood from 21 healthy subjects did not stain. After incubating MNC from these subjects with IFN α 2b for 48 hours, Mx proteins were detected in monocytes and lymphocytes. Within two days of starting treatment with subcutaneous IFN α 2b, granulocytes, monocytes and lymphocytes of 16 patients with cancer stained strongly for Mx proteins. The intensity of staining was correlated with the Mx content of whole blood measured using a specific ELISA. Prior to IFN treatment, cells from bone marrow and skin tissue specimens were negative for Mx proteins with the exception of endothelial cells. During treatment with IFN α 2b, nearly all cells from bone marrow and skin stained intensely.

Conclusions—These new monoclonal antibodies facilitate the detection of Mx positive cells in peripheral blood and in frozen or paraffin wax specimens. The advantage of this staining method is that individual cells which have responded to viruses or biologically active IFN α , β or ω can be identified.

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antiviral and immune modulatory activities.¹ Because of the short half-life of IFN in vivo and the existence of various IFN α subtypes, it has been difficult to quantitate endogenous production of IFN in humans.^{2,3} Biologically active IFN is not detectable in the circulation of healthy subjects⁴ and patients with bacterial infections.⁵ In contrast, a large number of patients with certain acute viral or autoimmune diseases, or both, have detectable serum IFN titres.^{4,6,7}

IFNs act on cells via specific cell membrane receptors.⁸ They induce the synthesis of a number of intracellular proteins—for example, the 2',5'-oligoadenylate synthetases, the 60 kD protein kinase (PKR), the Mx proteins, and β_2 -microglobulin. Of these IFN induced proteins, MxA and MxB are thought to have the highest specificity for type I IFNs.⁹⁻¹¹ Other IFN induced proteins can be induced by other cytokines, such as interleukin-6, tumour necrosis factor α , and IFN γ .^{9,12} MxA is also known to be a sensitive marker for type I IFN activity in vivo with a half-life in blood of over three days.¹³ The presence of MxA is sufficient and essential for the induction of resistance to influenza virus.^{14,15} The function of MxB is unknown.

The aim of this study was to establish a staining method for MxA and MxB in leucocytes and bone marrow and skin cells.

Methods

Peripheral blood from 21 blood donors and 17 patients with systemic lupus erythematosus (SLE) was collected in citrate, and blood smears and blood lysates (450 μ l blood plus 50 μ l lysis buffer) were prepared. The mononuclear cells were separated on Ficoll-Hypaque gradients. Blood samples and skin biopsy specimens were obtained from 16 patients with metastatic malignant melanoma before and seven to 14 days after the start of treatment with IFN. The treatment regimen comprised 3 \times 6 million IU rIFN α 2b/week. Bone marrow aspirates were obtained from 10 patients with chronic myelogenous leukaemia, five of whom had been treated with 5 million IU rIFN α 2/m² daily, and from five patients with non-haematological diseases.

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Type I interferons (IFN) are a family of closely related glycoproteins and proteins with potent

FIXATION

Blood smears were dried for 24 hours at room temperature and fixed in either cold methanol for 20 minutes or 0.05% glutaraldehyde for seven minutes. Bone marrow smears were fixed using the cold methanol method. The skin biopsy specimens were fixed overnight in 4% neutral buffered formalin.

PRODUCTION OF MONOCLONAL ANTIBODIES

Female balb/c mice were immunised repeatedly with 10 µg recombinant MxB and Mx1 proteins (days 0, 27, and 51) (kindly provided by Dr Pavlovic, Zürich, and Dr Julkunen, Helsinki). The first and second injections were given subcutaneously, and the third intravenously. On day 55, the mice were killed and their spleen cells fused to Sp2-0 myeloma cells at a ratio of 1:1 following published methods.^{15a} Supernatants of the cultured hybridomas were analysed using the screening test described below; positive cultures were cloned by limiting dilution. Selected hybridomas were expanded in culture and placed into a technomouse (Technomara, Fernwald, Germany). The antibodies recovered from the technomouse were purified by protein A chromatography.¹⁶

SCREENING TEST

Ninety six well plates were coated with 5 µg/ml recombinant Mx1, MxA (kindly provided by Dr Staeheli, Freiburg) or MxB for 16 hours at 4°C. After blocking non-specific binding with bovine serum albumin (BSA), the culture supernatants were added to the wells and incubated for 24 hours at room temperature. The plates were washed, sheep-anti-mouse-IgG coupled with alkaline phosphatase was added, and the plates were incubated for a further hour. P-nitrophenylphosphatase was used as the substrate.

SPECIFICITY TEST

rMxA, rMxB, rMx1, or lysates from IFN treated cells were electrophoresed on 2% SDS polyacrylamide gels and transferred to nitrocellulose filters using a semidry electroblotter. Non-specific binding was blocked with BSA and the various Mx-specific monoclonal antibodies were applied to the filters. After washing with TRIS/0.05% Tween, alkaline phosphatase conjugated goat anti-mouse antibody was added, and the blot was washed and stained with the alkaline phosphatase substrate 5-bromo-4-chloroindolyl-phosphate and nitroblue tetrazolium.

MX SPECIFIC ELISA

Aliquots (20 µl) of lysed citrated blood or cell suspensions were incubated for 16 hours at room temperature in 96-well plates (Maxisorb; Nunc, Roskilde, Denmark) which had been coated with the 2-95 monoclonal antibody (5 µg/ml) for 16 hours at 4°C. After washing, a biotinylated monoclonal antibody (5-237) directed against a second epitope on human Mx proteins was added. After two hours, the plates were washed and an avidin-alkaline phosphatase conjugate (Dianova, Hamburg, Ger-

many) was added for a further hour. After a final wash, the bound enzyme was revealed by means of a colour reaction at 405 nm using p-nitrophenylene-phosphate as substrate at pH 9.8 for 30 minutes. As an international standard for the two human Mx proteins is not available, Mx protein concentrations were expressed in laboratory units.¹⁷ One unit Mx is defined as the concentration of Mx protein present in 20 000 Wish cells treated with 1000 units rIFNα2b for 24 hours. Six dilutions of an internal standard were run in each assay.

STAINING OF PERIPHERAL BLOOD AND BONE MARROW CELLS

Blood and bone marrow smears were stained with the antibodies directed against the Mx proteins and with a Mx specific rabbit hyperimmune serum using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique, as described by Gala *et al.*¹⁸ The Mx specific rabbit hyperimmune serum was kindly provided by Dr Julkunen, Helsinki. Matched monoclonal antibodies (IgG1, IgG2a) were used as controls. Antibody bound alkaline phosphatase activity was visualised using Fast Red. Levamisole (2 mmol/ml) was added to all experiments to quench endogenous alkaline phosphatase activity. Slides were examined microscopically by two independent observers. The intensity of Mx staining was graded semi-quantitatively from - to +++.

STAINING OF SKIN TISSUE

Paraffin wax sections (3 µm) were dewaxed in xylene, rehydrated through an ethanol series and then washed in TRIS (pH 8.2). Paraffin wax sections or 3-5 µm snap frozen sections were mounted on coated glass slides to prevent the tissue sections from lifting during the immunohistochemical procedure. After blocking with 400 mg pure rabbit IgG/ml for one hour, the Mx monoclonal antibody (2-95) or the rabbit hyperimmune serum was added at a concentration of 0.5 µg/ml. After being incubated for 16 hours at 4°C in the case of the monoclonal antibody, the slides were washed and incubated consecutively with a rabbit anti-mouse antibody, a mouse antibody alkaline phosphatase complex, and finally with Fast Red (Dako, Glostrup, Denmark), each for 30 minutes. The procedure was performed twice. Sections were counter stained with haemalum and mounted with glycerine gelatine. Preincubation of the 2-95 monoclonal antibody (0.1 µg/ml) with rMxA at 5 µg/ml for two hours at room temperature completely abolished specific staining in skin, peripheral blood leucocytes, and bone marrow.

INCUBATION OF MONONUCLEAR CELLS WITH IFN

Purified mononuclear cells (1 million cells/ml) were incubated in 2 ml RPMI in the presence of 10% fetal calf serum at 37°C in 5% CO₂, either in the presence or absence of 100 IU IFNα2b/ml for two days. After 48 hours, cyto-centrifuge slides of these cells were prepared and dried in air for 24 hours before staining.

Table 1 Characteristics of Mx specific monoclonal antibodies

Clone	Isotype	Western blot				ELISA			APAAP
		rMxA	rMxB	nMxA/nMxB	rMx1	rMxA	rMxB	nMxA/nMxB	nMxA/nMxB
2-95	IgG1κ	+	+	+	+	+	+	+	+
5-111	IgG1κ	+	ND	-	+	ND	ND	ND	-
5-233	IgG1	+	ND	-	+	ND	ND	ND	-
5-237	IgG1κ	+	+	+	+	+	+	+	+
5-143	IgG2aλ	+	+	+	+	+	+	+	+
6-3	IgG1κ	+	-	+	-	+	ND	-	ND

The monoclonal antibodies were tested using a specific ELISA, western blotting and the APAAP technique on peripheral blood mononuclear cells.

ND = not done; + = positive signal; - = no signal.

Results

SPECIFICITY OF THE MX SPECIFIC MONOCLONAL ANTIBODIES

The 2-95, 5-237 and 5-143 monoclonal antibodies reacted with rMxA, rMxB, and lysates of IFN α treated cells, but not with those of non-treated cells. The protein bands had an expected molecular weight of about 80 kD. The same band pattern was seen when the blot was incubated with the Mx hyperimmune rabbit antiserum. Two monoclonal antibodies reacted only with rMxA and Mx1, but not with proteins from IFN treated cells. One monoclonal antibody reacted with rMxA and proteins from IFN treated cells, but not with rMxB. The subsequent staining experiments were performed using the protein A purified 2-95 (table 1) and 5-143 monoclonal antibodies, and the rabbit hyperimmune serum.

STAINING OF PERIPHERAL BLOOD CELLS IN VITRO

Granulocytes, monocytes and lymphocytes extracted from freshly collected blood from 21 healthy subjects were negative when stained with the 2-95 and 5-143 monoclonal antibodies. However, after incubating these cells with IFN α for 48 hours, monocytes and lymphocytes stained intensely (data not shown). Coincubation of the antibody with rMxA abolished the specific staining pattern. This pattern was seen in blood from all 21 subjects. Single, small positive granules were seen in the cytoplasm of non-cultured granulocytes from the same subjects, even in the presence of levamisole. These granules were also positive when stained in the absence of a specific monoclonal antibody. These granules probably contain unquenched alkaline phosphatase activity. Both fixation methods (glutaraldehyde, cold methanol) gave the same results.

STAINING OF PERIPHERAL BLOOD CELLS IN VIVO

After demonstrating specific Mx staining of mononuclear cells cultured in the presence of IFN α , leucocytes from patients undergoing

treatment with IFN α were studied (fig 1). Leucocytes from 16 patients with metastatic malignant melanoma were stained prior to treatment. Table 2 summarises the appearance of Mx proteins in the circulating leucocytes after injecting 6 million IU rIFN α 2b over time. Four hours after the injection, monocytes expressed detectable Mx proteins. Granulocytes stained positively after six hours. Lymphocytes stained positively after 24 hours. Coincubation of the 2-95 monoclonal antibody with rMxA almost abolished staining in granulocytes and mononuclear cells, demonstrating the specificity of the reaction. To quantify the amount of Mx proteins in peripheral blood leucocytes, whole blood was lysed and subsequently analysed using a Mx specific ELISA. Consistent with the staining results, the concentration of Mx proteins in peripheral blood rose within 48 hours of the start of treatment with IFN α in patients with cancer (table 2).

STAINING OF LEUCOCYTES FROM PATIENTS WITH SLE

Most of the 17 patients with SLE had detectable concentrations of endogenously produced IFN in their peripheral blood. Two patients had normal blood Mx protein concentrations but their leucocytes were negative on staining with the 2-95 monoclonal antibody. The remaining 15 patients had increased blood Mx protein concentrations and Mx positive leucocytes. Granulocytes and monocytes from these patients also stained positively. Similar results were obtained with the 5-143 monoclonal antibody.

STAINING OF BONE MARROW CELLS

Bone marrow smears from five patients with non-haematological diseases and 10 patients with CML, five of whom were being treated with IFN, were studied. Bone marrow cells were Mx negative in patients not receiving IFN treatment. Alkaline phosphatase containing granules were seen in 1-10% of the mature

Table 2 Induction of Mx protein expression in leucocytes from patients with malignant melanoma before and after the first subcutaneous injection of 6 million IU IFN α 2b measured using an Mx specific ELISA or the APAAP technique

Cell type	Hours after injection of IFN α 2b						
	0	2	4	6	12	24	48
Monocytes	-	-	+	+	++	++	+++
Granulocytes	-	-	+	+	+++	+++	+++
Lymphocytes	-	-	-	-	+	+	++
ELISA*	1.2 (0.8)	1.6 (2.1)	2.3 (1.7)	4.6 (2.2)	8 (2.9)	15 (3.6)	18 (4.1)

*Expressed as mean (SD) mU/1000 leucocytes.

The concentration of Mx protein in healthy subjects varies between 0.6 and 2.4 mU/1000 leucocytes.

- = Negative; + = mildly positive; ++ = moderately positive; +++ = strongly positive.

granulocytes. Bone marrow aspirates taken from patients being treated with IFN α 2b at a daily dose of 5 million IU/m² were strongly Mx positive. Nearly all bone marrow cells stained intensely for Mx (fig 2). Megakaryocytes could be identified easily as Mx positive cells in the smears.

STAINING OF SKIN BIOPSY SPECIMENS

Skin biopsy specimens from 16 patients with metastatic malignant melanoma were stained before and during treatment with IFN α 2b (fig 3). Prior to treatment, keratinocytes from eight patients were mildly Mx positive when stained with 2 μ g 2-95 monoclonal antibody/ml. When the same tissue specimens were stained with this antibody at a concentration of 0.5 μ g/ml, keratinocytes from only one patient gave a Mx positive signal. However, nearly all vessels had

mildly positive endothelial cells. In skin biopsy specimens taken during the 3 \times 6 million IU/week IFN α 2b treatment regimen, keratinocytes, endothelial cells, and cells in sweat and sebaceous glands were strongly Mx positive on staining with the 2-95 monoclonal antibody at a concentration of 0.5 μ g/ml. Occasional smooth muscle cells were also positive for Mx. The Mx staining pattern was similar in frozen and paraffin wax sections. Preincubating the 2-95 monoclonal antibody with rMxA completely abolished specific staining.

Discussion

Using the monoclonal antibodies generated in our laboratory, which recognise MxA and MxB, we successfully stained IFN induced Mx proteins in peripheral blood leucocytes and bone marrow and skin cells.

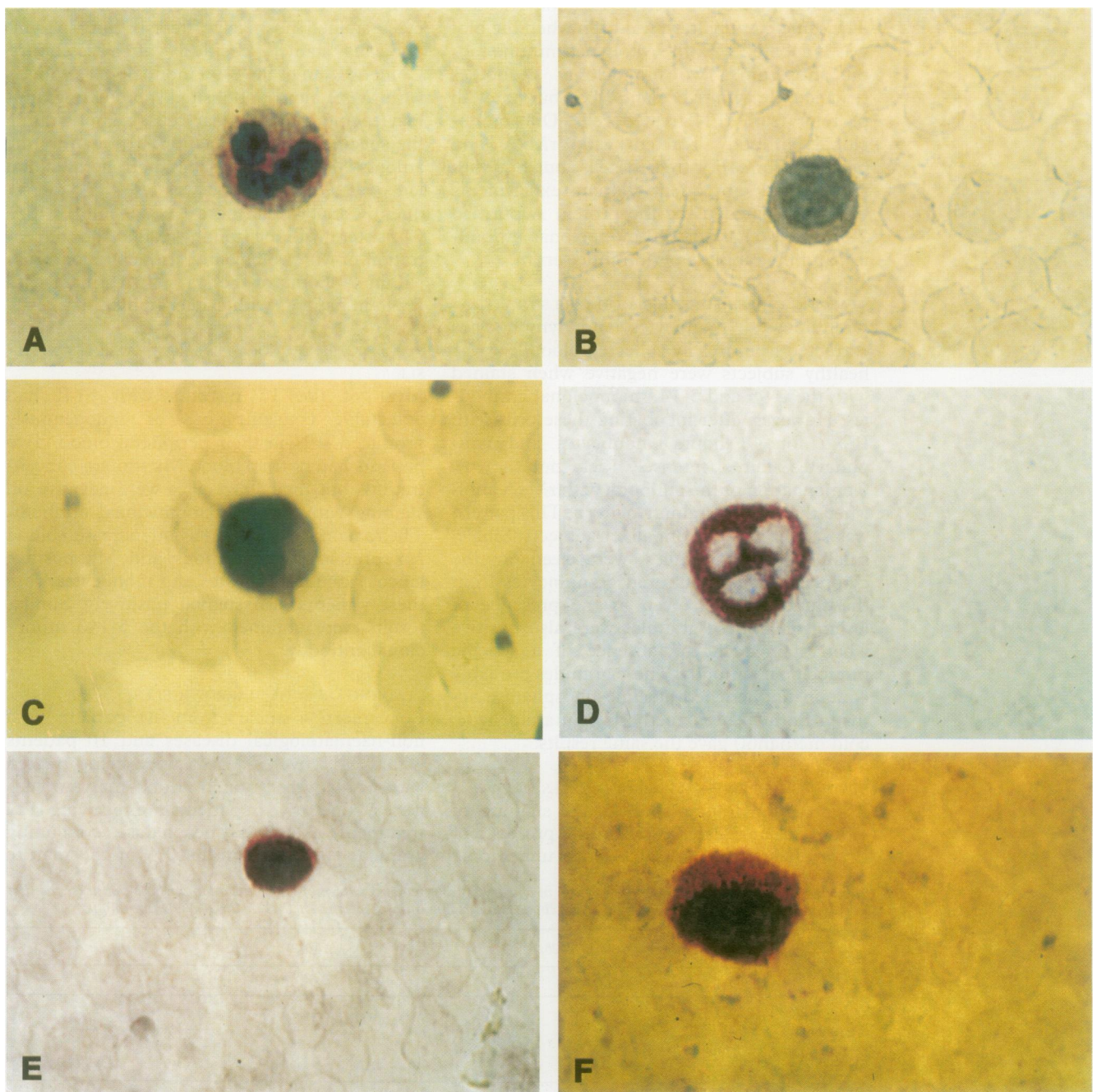


Figure 1 Immunostaining (APAAP) of Mx proteins in leucocytes (granulocyte (A), lymphocyte (B), monocyte (C)) from a patient with malignant melanoma prior to (negative control) and during (granulocyte (D), lymphocyte (E), monocyte (F)) treatment with IFN α 2b (3 \times 6 million IU/week).

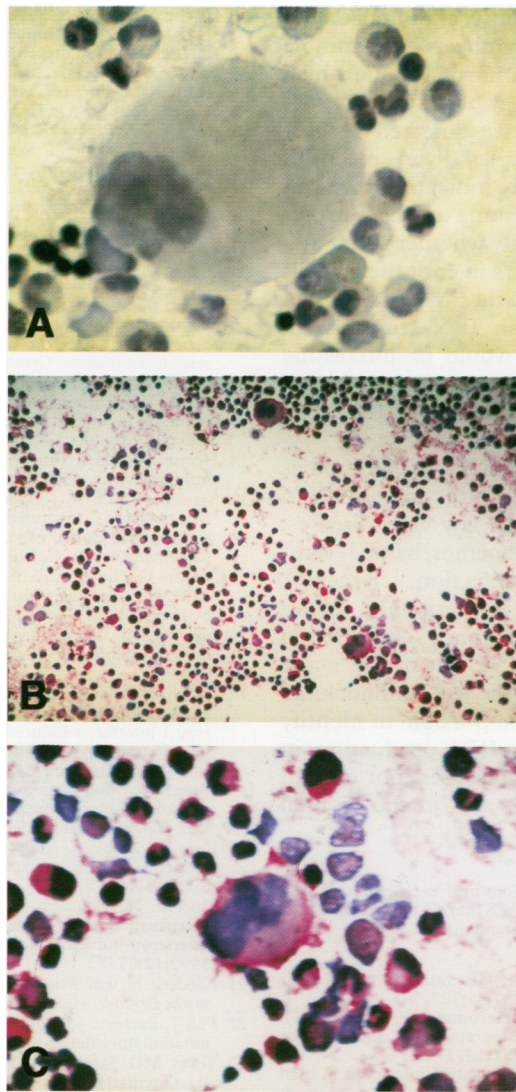


Figure 2 Immunostaining of Mx proteins in bone marrow aspirates. Bone marrow from a patient with CML prior to (A) and during treatment with IFN α 2b (5 million/m 2) at low (B) and high (C) magnification.

As anticipated, nearly all of the cells investigated stained positively when pretreated with IFN α either in vitro or in vivo. Erythrocytes were always Mx negative. In contrast to the results of Ronni *et al.*,¹⁹ we found that granulocytes also expressed Mx proteins. This discrepancy may be explained by the fact that Ronni *et al.* examined MxA expression in granulocytes treated in vitro by IFN, whereas we stained these cells in blood smears from untreated and treated patients. Prior to treatment with IFN, weakly positive granules containing alkaline phosphatase positive granules were observed. However, after a subcutaneous injection of IFN α , granulocytes became intensively positive for Mx.

The fact that in the presence of type I IFNs granulocytes, monocytes and lymphocytes express Mx is of major importance for measuring Mx proteins in whole blood. In order to relate the concentration of Mx proteins in whole blood to the number of Mx positive cells within a single blood sample, it is necessary to know which cell types and how many leucocytes are Mx positive. As Towbin *et al.*²⁰ reported for MxA, more than 90% of the circulating lymphocytes and monocytes are Mx positive in

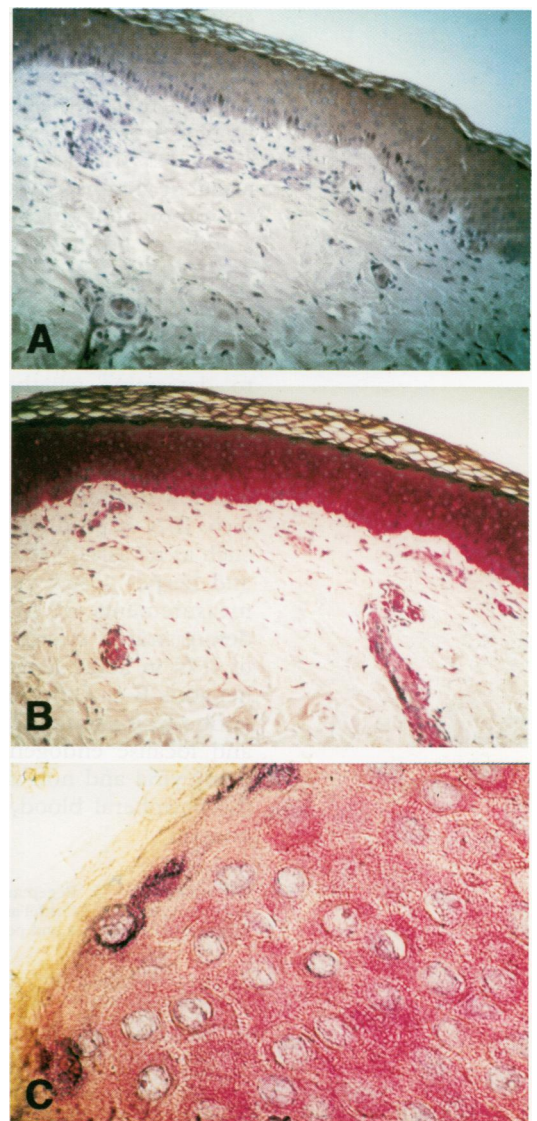


Figure 3 Immunostaining of Mx proteins in skin biopsy specimens from a patient with malignant melanoma prior to (A) and during treatment with IFN α 2b (3 \times 6 million IU/week) at low (B) and high (C) magnification.

patients with endogenous IFN production and in those undergoing treatment with IFN. In addition, almost all granulocytes are Mx positive. Therefore, the concentration of Mx protein in whole blood should be calculated as unit Mx proteins per 1000 leucocytes.

Cells other than leucocytes also respond to type I IFN.²¹ Staining of skin and bone marrow cells showed that nearly every cell in these organs has the capacity to produce either MxA or MxB, or both, after stimulation with IFN. Even very immature cells, such as blasts or promyeloblasts, synthesise Mx protein after the appropriate stimulus. Unstimulated, normal bone marrow cells do not express Mx protein. Therefore, appreciable amounts of biologically active type I IFNs are not present in normal bone marrow.

In skin biopsy specimens, only endothelial cells stained positively at low antibody concentrations (0.5 μ g/ml). During treatment with IFN α , keratinocytes, fibroblasts, smooth muscle cells, endothelial cells and cell in sweat glands express large amounts of Mx protein. F \ddot{a} h *et al.* stained skin biopsy specimens with an Mx1 specific antibody and observed a similar

staining pattern.²² Apparently, endothelial cells are the only cells which synthesise measurable amounts of Mx protein in normal tissue which suggests that these cells may produce low levels of type I IFNs. Tovey *et al* detected specific IFN mRNA in tissue samples (liver, spleen) of accident victims,²³ possibly indicating continuous production of IFN in some tissues. Further studies are needed to clarify whether spontaneous IFN production occurs in endothelial cells of healthy subjects or whether MxA is synthesised independently of an IFN stimulus. In autoimmune diseases, it would be interesting to determine whether endothelial cells are responsible for the high endogenous IFN concentrations found in these patients.

The 2-95 and 5-143 monoclonal antibodies can now be used to study production of IFN *in vivo*. IFN is produced in a few viral diseases, such as measles,²⁴ HIV,²⁵ haemorrhagic fever,²⁶ and herpes zoster virus infection.²⁷ Recently, measurements of MxA in peripheral blood demonstrated strong endogenous production of IFN in acute hepatitis A, but not in acute hepatitis B or C.¹⁷ Using the 2-95 monoclonal antibody, it should now be possible to identify and localise endogenous IFN production in many viral and non-viral diseases, not only in the peripheral blood, but also in the visceral organs.

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