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EFFECTS OF MURINE RECOMBINANT INTERFERON-GAMMA ON RAT LIVER FAT STORING CELL PROLIFERATION, CLUSTER FORMATION AND PROTEOGLYCAN SYNTHESIS

AXEL M. GRESSNER* and MICHAEL ALTHAUS
Department of Clinical Chemistry and Central Laboratory, University of Marburg, 3550 Marburg, Baldingerstr., Federal Republic of Germany

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Abstract—Rat liver fat storing cells (FSC, perisinusoidal lipocytes, Ito cells) in primary culture were exposed to various concentrations of murine recombinant interferon-gamma (rIFN-γ) in the range of 1 to 50 units/mL medium for 72 hr. FSC kept in complete medium (10% fetal calf serum) showed a dose-dependent increase of both [^{3}H]thymidine incorporation (up to 2.3-fold) and DNA content of culture. Reverse (inhibitory) effects were obtained with cells kept under serum-reduced (0.5% fetal calf serum) conditions. The synthesis of medium proteoglycans and of total cellular protein was not affected by rIFN-γ. By bromodeoxyuridine-staining (BrdUrd) and phase contrast microscopy it is shown that rIFN-γ stimulates strongly the cluster growth of FSC in culture. The cluster forming cells differ in their morphology and their cytoskeleton-staining from typical FSC. They were found to be mostly desmin and α-actin negative or weakly positive but highly proliferative. Because no contaminating fibroblasts and other cell types were detected in any appreciable amounts in the early cultures we conclude that the clustered cells might be a rapidly proliferating subpopulation of FSC, which is promoted by rIFN-γ.

Fat storing cells (FSC†), also known as Ito cells, perisinusoidal lipocytes or vitamin A storing cells [1], are localized near the sinusoidal surface of the parenchymal liver cells beneath the endothelial cell layer in the space of Disse [2, 3]. Apart from their primary role in the storage and metabolism of retinoids [4], the cells are likely to play an important role in the pathogenesis of liver fibrosis because they have been identified to be capable of synthesizing and secreting several connective tissue components [5]. The ability of cultured fat storing cells to synthesize collagen [6], proteoglycans, structural glycoproteins, and hyaluronan [7] is well documented. The pattern of glycosaminoglycans synthesized by fat storing cells is similar to that in the fibrotic liver extracellular matrix [5, 8, 9]. The cells are able to proliferate and transform via transitional cells into myofibroblast-like cells in areas of necrosis and inflammation [10, 11].

Interferon-gamma (IFN-γ) is recognized as a mediator playing a pivotal role in the immune system due to a broad range of immune modulatory, antiviral, antitumor, and antiproliferative effects [12–14]. It is secreted by activated T-lymphocytes and probably also by natural killer cells [15]. Detailed studies deal with the influence of IFN-γ on cell growth and differentiation [16]. Most of the studies carried out with crude or partially purified IFN-γ report on an inhibition of cell growth under the influence of IFN-γ on tumor cells and normal cell lines [16], some cell types appeared to be resistant to the action of IFN-γ [17]. A contamination with traces of other lymphokines could not be excluded, thus leaving the possibility that the growth inhibitory effect may be partially due to the presence of those molecules because IFN-γ has a strong synergistic effect with other lymphotoxins [18]. rIFN-γ causes a specific decrease of collagen synthesis in cultured fibroblasts and synovial cells [19–23] and inhibits collagen synthesis *in vivo* in the mouse [24]. The influence of recombinant IFN-γ on cell growth, especially on fibroblasts, remains controversial, some authors describe an inhibition [25], others stimulation [26–28], and even one study reports on a bidirectional modulation of fibroblast growth [29]. Thus, the introduction of rIFN-γ did not solve the problem and the controversy about the effect of rIFN-γ on growth of diploid fibroblasts. Culture conditions and the source of the cells might be relevant determinants.

Up to now, there are only few studies which deal with the influence of rIFN-γ on proteoglycan synthesis [30, 31] and there is no information available concerning the effect of rIFN-γ on fat storing cells, the main connective tissue producing cell type in liver. However, there are indications that IFN-γ may be useful in the treatment of liver cirrhosis [32] and studies have been designed to determine the effectiveness of treatment of chronic hepatitis B virus infection with rIFN-γ [33, 34].

* Correspondence to: Prof. Dr Axel M. Gressner, Abt. für Klinische Chemie, und Zentrallaboratorium, Klinikum der Philipps-Universität, Baldingerstraße, 3550 Marburg, F.R.G.

† Abbreviations: BrdUrd, bromodeoxyuridin; CHAPS, 3 - [(3 - cholamidopropyl)dimethylammonio] - 1 - propanesulfonate; FCS, fetal calf serum; FSC, fat storing cells; PBS, phosphate buffered saline; PMSF, phenyl-methyl-sulfonyl-fluoride; NEM, *N*-ethylmaleimide; rIFN-γ, murine recombinant interferon-gamma; TCA, trichloroacetic acid.

In the present study we have looked for the influence of rIFN-γ on proliferation and proteoglycan synthesis of fat storing cells kept under various conditions *in vitro* to obtain information on the basic effects of rIFN-γ on those cells involved primarily in the pathogenesis of liver fibrosis.

MATERIALS AND METHODS

Isolation and culture of fat storing cells

Fat storing cells were prepared from 1-year-old male Sprague–Dawley rats (body weight 500–700 g, Lippische Versuchstierzucht, Extertal, F.R.G.), which had free access to a standard laboratory chow diet containing 15 I.U. vitamin A/kg and tap water. Non-parenchymal liver cells were isolated by the pronase–collagenase method [35] in a sequence of non-circulating and recirculating perfusions with slight modifications described in detail elsewhere [8]. Fat storing cells were purified from the non-parenchymal cell suspension by a single step density gradient centrifugation with Nycodenz (analytic grade, Nyegaard Co. AS, Oslo, Norway), which is reported in detail previously [8]. The cells were identified by their typical light microscopic appearance [35], transmission electron microscopy [35], immunofluorescence staining for desmin and vimentin [36], vitamin A-specific autofluorescence [8] and, negatively, by the inability to phagocytose latex beads (LB11 polystyrene beads, mean diameter 1.1 μm), to stain for peroxidase and to express Fc receptors [37, 38]. The mean purity of freshly isolated cells was 80 ± 7%, cell viability as checked by Trypan blue exclusion was 91 ± 6%, and the yield ranged from 15 to 43 × 10^6 cells/liver. The cells were seeded with a density of 0.4 to 2 × 10^5 cells/cm^2 and maintained as monolayers in 6-well or 24-well culture plates (Greiner, Nürtingen, F.R.G.) with Dulbecco's modification of Eagle's medium (DMEM) containing 4 mmol/L L-glutamine, penicillin (100 I.U./mL), streptomycin (100 μg/mL) and 10% (v/v) fetal calf serum (all from Boehringer GmbH, Mannheim, F.R.G.). The cells were cultured in a humidified atmosphere of 5.5% CO_2–94.5% air. The first change of medium was made 6 hr after seeding; the second 12 hr later after which the purity of fat storing cells was higher than 95%.

Determination of proteoglycan synthesis

Recombinant murine rIFN-γ was generously provided by Dr G. R. Adolf (Ernst Boehringer Institute for Drug Research, Vienna, Austria). The specific activity was 1 × 10^7 units/mg (bioassay: mouse L cells/EMCV) and the level of endotoxin was 0.032 units/mg. The rIFN-γ was dissolved in 0.154 M NaCl, aliquots were diluted with medium (1:10^4–1:10^6) and stored at −40°. The synthesis of sulfated proteoglycans was determined by the incorporation of [^{35}S]sulfate (18.5–22.2 GBq/mmol; 740 kBq/mL medium; New England Nuclear Corp., Boston, MA, U.S.A.) into the glycosaminoglycans during a labeling period of 24 hr (in combination with the incorporation of [^{3}H]thymidine (see below) as double labeling). Labeled proteoglycans were determined only in the medium, because fat storing cells were shown to secrete nearly 80% of newly synthesized proteoglycans into the culture medium [8]. The medium was removed by pipetting and immediately centrifuged (6 min, 1000 *g*, 4°). Seven hundred μL of the cell-free medium were mixed with 3 mL buffer A (7 M urea, 0.13 M Tris–Cl, 1 mM EDTA, 1 mM PMSF, 10 mM NEM, 0.1% CHAPS, pH 7.5) and unlabeled glycosaminoglycans (hyaluronic acid, heparin, chondroitin-4-sulfate, chondroitin-6-sulfate; all from Sigma Chemical Co., Munich, F.R.G.) were added as carrier. Then the proteoglycans were bound during an incubation (1 hr, 4°) to a batch of DEAE-sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the same buffer. After washing the resin seven times with buffer B (= buffer A + 0.1 M NaCl + 10 mM thymidine, pH 7.5) total proteoglycans were eluted with buffer C (= buffer A + 0.8 M NaCl, pH 7.5). An aliquot of the supernatant was mixed with Instagel (Packard Instruments Co., Downers Grove, U.S.A.) and counted for radioactivity in a Packard scintillation counter (Minaxi type 4430). No interference from [^{3}H]thymidine was noticed.

Determination of specific types of proteoglycans

For analysis of the specific types of glycosaminoglycan chains total proteoglycans were subjected to consecutive degradations with nitrous acid to yield the incorporation of [^{35}S]sulfate into heparan sulfate and to enzymatic digestions with chondroitin AC- (E.C. 4.2.2.5) and -ABC-lyases (E.C. 4.2.2.4) (Seikagaku Fine Chemicals, Tokyo, Japan), to obtain the fractions of chondroitin-4,6-sulfate and dermatan sulfate, respectively. Details of the method have been reported previously [8].

Measurement of [^{3}H]*thymidine incorporation into DNA and of* [^{3}H]*thymidine uptake*

DNA synthesis was determined by incubation of the cells with [6-^{3}H]thymidine (673 GBq/mmol, 18.5 kBq/mL medium, New England Nuclear Corp.) during a labeling period of 24 hr (simultaneously with [^{35}S]sulfate [see above] as double labeling). The medium was poured off and the cell layer was washed twice each with 2 mL phosphate-buffered saline. Cells were harvested by three cycles of freezing (with liquid nitrogen) and thawing and suspended in buffer (50 mM sodium phosphate, 2 M NaCl, pH 7.4). An aliquot of this suspension was mixed with bovine serum albumin as carrier and with ice-cold trichloroacetic acid (TCA). After precipitation (2 hr, 4°) the sample was centrifuged and the sediment was washed by dissolution in 0.5 M NaOH and reprecipitation with trichloroacetic acid, repeating the cycles four times. The final sediment was solved in NaOH mixed with Instagel and counted for radioactivity.

The uptake of [^{3}H]thymidine in a TCA-soluble fraction (cytoplasmic pool) was measured by labeling the cells for various times with [^{3}H]thymidine as described. The incubation was stopped by washing the cell layer five times with ice-cold PBS containing 10 mM thymidine. The further steps are exactly as described above. The supernatant of the TCA-precipitation was mixed with Instagel and counted for radioactivity. In the final step no radioactivity from the labeled proteoglycans was detectable.

Measurement of cellular protein synthesis

Cells were exposed for 24 hr to L-[3,4-^{3}H]valine (2150 GBq/mmol, 185 kBq/mL medium, New England Nuclear). The radioactivity of total cellular protein was determined by sequential extraction following the method of Mans and Novelli [39] and referred to the DNA content of the cultures.

Cell staining procedures

Crystal violet. The cell layer was washed three times with cold PBS and stained with 0.5% crystal violet in methanol/water (1:4, v/v) for 15 min. Excess dye was removed by washing the cell layer with water several times [40].

Indirect immunoperoxidase staining of desmin, vimentin, and α-actin. At the end of the culture time the cells were washed with PBS and fixed with ethanol/acetic acid 95:5 (v/v) for 15 min. Thereafter the cells were washed again and incubated with FCS/PBS 50:50 (v/v) for 15 min at 37° to block non-specific binding sites. After washing the cells three times with PBS they were incubated with monoclonal mouse antibodies to human desmin, vimentin (both from Dakopatts, Glostrup, Denmark), and smooth muscle α-actin (Boehringer, Mannheim, F.R.G.), respectively, for 45 min at 37°. Then the cells were washed four times and incubated with peroxidase-conjugated rabbit antimouse-immunoglobulin (Dakopatts) for further 45 min at 37°. After excessive washing with PBS cells were stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) using hydrogen peroxide (3%), nickel chloride, and cobalt chloride (3%) as intensifier.

Bromodeoxyuridin incorporation. Cells were labeled for 24 hr with BrdUrd (5×10^{-5} M final concentration, Amersham Buchler, Braunschweig, F.R.G.). After washing with PBS cells were fixed with ethanol/acetic acid 95:5 (v/v) and incubated with anti-BrdUrd-antibody (Dakopatts) and nuclease (Amersham Buchler, Braunschweig, F.R.G.). Peroxidase-conjugated antimouse immunoglobulin (Dakopatts) was used as second antibody. Nuclei which had taken up BrdUrd were stained with DAB. Details of the method have been described recently [41].

General techniques

Cells were quantitated by fluorometric determination of DNA [42] using calf thymus DNA (type I, Sigma Chemical Co., Munich, F.R.G.) as a standard. Viability was assayed by estimation of the percentage of unstained cells when incubated with 0.25% Trypan blue in phosphate-buffered saline.

Statistical analysis

Differences of independent determinations were tested with the Scheffé multiple range test for pairwise comparisons of means; $P < 0.05$ was accepted to be statistically significant [43]. In cases of expression of results as ratios of original values each divided by the corresponding control value the significance of differences was tested with the original data.

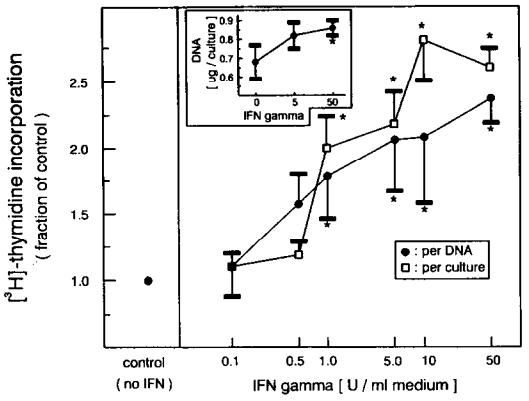

Fig. 1. Effect of rIFN-γ on the DNA content per culture well and [^{3}H]thymidine incorporation into DNA of fat storing cells (seeding density 0.4×10^5 cells/cm^2). Cells were kept in medium supplemented with 10% FCS and then incubated without (control) and with rIFN-γ for further 72 hr. Labeling with 18.5 kBq [^{3}H]thymidine/mL medium was carried out during the last 24 hr of culture. Mean values ± SD of 6–9 experiments are shown. *Statistically significant.

RESULTS

Effect of rIFN-γ on [^{3}H]thymidine incorporation and uptake

Beginning with the first day of culture fat storing cells were exposed to various concentrations of rIFN-γ for the following 72 hr. After the first 48 hr of the IFN incubation period the medium was removed and fresh medium containing rIFN-γ and [^{3}H]thymidine was added for the final 24 hr. The cell viability at the end of the incubation time was >95% for untreated cultures and cells exposed to 50 units rIFN-γ, respectively. The [^{3}H]thymidine incorporation related to cellular DNA shows a dose-dependent increase under the influence rIFN-γ as it was the case for the total DNA content per culture (Fig. 1). A concentration equal to or higher than 1 unit rIFN-γ/mL caused a significant increase of [^{3}H]thymidine incorporation, doubling of the incorporation rate was obtained at 5 units/mL, if the experiments were carried out in the presence of 10% fetal calf serum in the culture medium. Using fat storing cells kept in medium containing 0.1% fetal calf serum for 48 hr and then supplemented with 10% fetal calf serum in the presence and absence of rIFN-γ showed also a significant increase of [^{3}H]thymidine incorporation per DNA at concentrations of 5 and 10 units/mL medium, respectively (Table 1). Thus, the serum-induced activation of fat storing cells is promoted by rIFN-γ. We also measured the effect of rIFN-γ on slowly growing fat storing cells. The cells were allowed to grow initially in medium containing 10% fetal calf serum for 72 hr, after which time the cells were carefully washed and cultured further with 0.5% fetal calf serum for another 48 hr period. Thereafter rIFN-γ was added for a further 72 hr period. Figure 2 shows that rIFN-γ significantly inhibits [^{3}H]thymidine incorporation in a dose-related manner and reduces the increase of DNA content per culture without affecting cell viability in

Table 1. Effect of rIFN-γ on the incorporation of [^{3}H]thymidine into DNA during activation of fat storing cells by addition of fetal calf serum

IFN-α (units/mL medium)	[^{3}H]Thymidine incorporation per DNA (fraction of control)
0	1.00 ± 0.14
0.5	1.03 ± 0.10
1.0	1.14 ± 0.08
5.0	1.34 ± 0.14*
10.0	1.54 ± 0.17*

The cells (seeding density 0.4×10^5 cells/cm^2) were kept in medium containing 0.1% FCS for 48 hr and then supplemented with 10% FCS in presence and absence (control) of rIFN-γ. Labeling with [^{3}H]thymidine was carried out as described in Fig. 1. Mean values ± SD of 3 experiments are listed.

* Statistically significant.

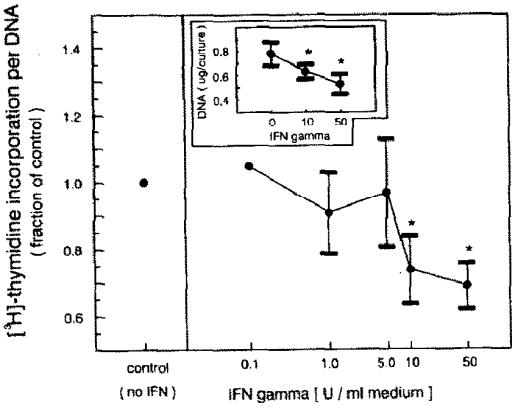

Fig. 2. Effect of rIFN-γ on the DNA content per culture well and [^{3}H]thymidine incorporation into DNA of fat storing cells (seeding density 0.4×10^5 cells/cm^2). Cells were kept under fetal calf serum-depleted conditions (0.5% FCS) for 48 hr and then incubated without (control) and with rIFN-γ for further 72 hr. The cells were labeled with 18.5 kBq [^{3}H]thymidine/mL medium during the last 24 hr. Mean values ± SD of 4–8 experiments are shown. *Statistically significant.

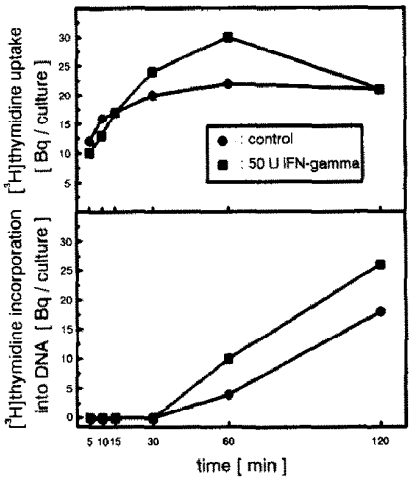

Fig. 3. Effect of rIFN-γ on the cellular uptake of [^{3}H]thymidine and [^{3}H]thymidine incorporation into DNA of fat storing cells. Cells were kept under the same conditions as described in Fig. 1 and were exposed to rIFN-γ (0 and 50 units/mL, respectively) for 60 hr. The cells were labeled with 18.5 kBq [^{3}H]thymidine at the third culture day. [^{3}H]Thymidine uptake and incorporation were determined from 5 to 120 min after addition of label as described in Materials and Methods. Mean values of a duplicate determination are shown.

comparison to the control not receiving rIFN-γ. We tested the dependence of the rIFN-γ effect on different cell densities using seeding densities of 0.4 and 1.0×10^5 cells/cm^2, respectively. Cells were exposed to rIFN-γ beginning at the first day for 72 hr in fully supplemented medium as described. Both groups show identical dose-dependent increases of [^{3}H]thymidine incorporation in response to rIFN-γ. A maximum stimulation was observed at a concentration of 50 units rIFN-γ/mL medium (2.3-fold increase of [^{3}H]thymidine incorporation in both groups; data not shown). To evaluate possible changes in the cellular transport of [^{3}H]thymidine in rIFN-γ treated cells we compared the rate of [^{3}H]thymidine uptake into the cytosol of treated (50 units rIFN-γ/mL) and control cultures (both in 10% fetal calf serum). Figure 3 shows only a sight increase of the uptake under influence of rIFN-γ after 60 min, but identical values were obtained after 120 min. [^{3}H]Thymidine incorporation into DNA (acid insoluble fraction), however, shows an increase under the influence of rIFN-γ after 60 and 120 min (Fig. 3).

To get further information on the effect of rIFN-γ on the incorporation of [^{3}H]thymidine we added hydroxyurea (10 mM) to cultures (0, 5, and 50 units rIFN-γ/mL medium; 10% FCS) 3 hr before labeling with [^{3}H]thymidine. Under this condition proliferative DNA-synthesis is selectively inhibited leaving DNA repair synthesis unchanged [44]. Table 2 shows no significant differences between rIFN-γ treated and control cultures in the presence of hydroxyurea. Only a small fraction of [^{3}H]thymidine is used for DNA repair in both groups, whereas cultures which did not receive hydroxyurea show enhanced [^{3}H]thymidine incorporation under the

Table 2. Effect of rIFN-γ on [^{3}H]thymidine incorporation in the presence of hydroxyurea

rIFN-γ (units/mL medium)	[^{3}H]Thymidine incorporation (kBq/culture)	
	with hydroxy-urea	without hydroxy-urea
0	4.4 ± 0.5	144 ± 7
5	3.4 ± 0.2	239 ± 27*
50	3.9 ± 0.6	289 ± 56*

Cells were exposed for 72 hr to rIFN-γ as described in Fig. 1. Three hours before labeling with [^{3}H]thymidine hydroxyurea was added (10 mM). Mean values ± SD of 4 experiments are shown.
* Statistically significant.

influence of rIFN-γ as described above (Fig. 1). Thus stimulated [^{3}H]thymidine incorporation represents cell replication rather than increased DNA repair synthesis.

Influence of rIFN-γ on [^{3}H]*valine incorporation into total protein*

Fat storing cells kept in medium containing 10% FCS were labeled with [^{3}H]valine in the absence and presence of rIFN-γ (up to 50 units/mL medium) during the last 24 hr of culture. Cells treated with rIFN-γ for 72 hr had a total cellular protein synthesis which is similar to that of control cultures (2.1 ± 0.21 vs 1.9 ± 0.18 MBq/mg DNA at 50 units/mL rIFN-γ).

Effect of rIFN-γ on total and specific proteoglycan synthesis

The effect of rIFN-γ on the synthesis of proteoglycans was tested by exposing fat storing cells at the first day of culture to various concentrations of the lymphokine (0.1–50 units/mL) for further 72 hr. Medium was changed after 48 hr and fresh medium was added containing new rIFN-γ and [^{35}S]sulfate for 24 hr. No significant change of total [^{35}S]sulfate labeled proteoglycan synthesis was noticed in the range of 0.1 to 50 units/mL rIFN-γ in the medium (not shown). From these samples the pattern of specific medium proteoglycans was analysed, which shows that dermatan sulfate is the major fraction, followed by chondroitin sulfate and a small portion of heparan sulfate. Proteoglycans of rIFN-γ treated fat storing cells (50 units/mL) show no significant variation of this distribution profile (data not shown).

Effect of rIFN-γ on cluster formation

During the initial days of primary culture fat storing cells are homogenous in phase contrast microscopic appearance and size. The purity of these cultures is >98% and only a few contaminating Kupffer cells and endothelial cells but no fibroblasts could be detected. Beginning with the third culture day cluster forming cells could be observed by phase contrast microscopy and cytoskeleton staining. These cells appear smaller than typical fat storing cells, the cell shape is longish and the nucleus is clearly visible but only a few fat droplets are detectable and, thus, vitamin A fluorescence is reduced. At the seventh/eighth day of culture the major fraction of normally growing fat storing cells shows positive vimentin-, desmin- and α-actin-staining (Fig. 4). Fractions of 0.07 and 0.16 of these non-clustered cells, however, are definitely desmin- and actin-negative, respectively. Cluster forming cells are positive for vimentin but are mostly negative or only weakly positive for desmin- and α-actin (Fig. 4). To test the proliferation activity of both cell types we measured immunocytochemically the incorporation of BrdUrd into cell nuclei. Figure 5 shows that all cell clusters are positively stained at the eighth culture day. The microscopic picture shows numerous positively stained nuclei of cluster forming cells but only few positive nuclei of non-clustered fat storing cells (Fig. 5). Thus cluster forming cells seem to be the principal proliferating cells in these cultures. To test the influence of rIFN-γ on cluster formation, fat storing cells were seeded with a density of 0.2×10^5 cells/cm^2 and kept with fully supplemented medium. rIFN-γ (50 units/mL medium) was added at the first day of culture. Medium containing rIFN-γ was renewed every 48 hr. The number of clusters was counted at various culture times by phase-contrast microscopy or by crystal violet-staining. Table 3 shows a time-dependent increase of the number of clusters in rIFN-γ exposed cultures beginning with the third day of culture. Thus rIFN-γ seems to stimulate the formation of clusters of a highly proliferative subpopulation of cultured fat storing cells. This can be seen even by macroscopic examination of the culture plates having a significant number of foci in rIFN-γ exposed cultures.

DISCUSSION

This study was designed to analyse *in vitro* some basic effects of murine rIFN-γ on the major matrix producing cell type in liver, i.e. the fat storing cell [5–7]. Our data show an effect neither on the rate of total medium proteoglycan synthesis nor on the distribution profile of chondroitin sulfate, dermatan sulfate and heparan sulfate. Furthermore, general cellular protein synthesis was not changed either.

rIFN-γ changed the mitotic activity of cultured fat storing cells in a dose-dependent and complex manner. Under serum-reduced conditions rIFN-γ inhibited the rate of [^{3}H]thymidine incorporation but the opposite effect, i.e. enhanced proliferation, was

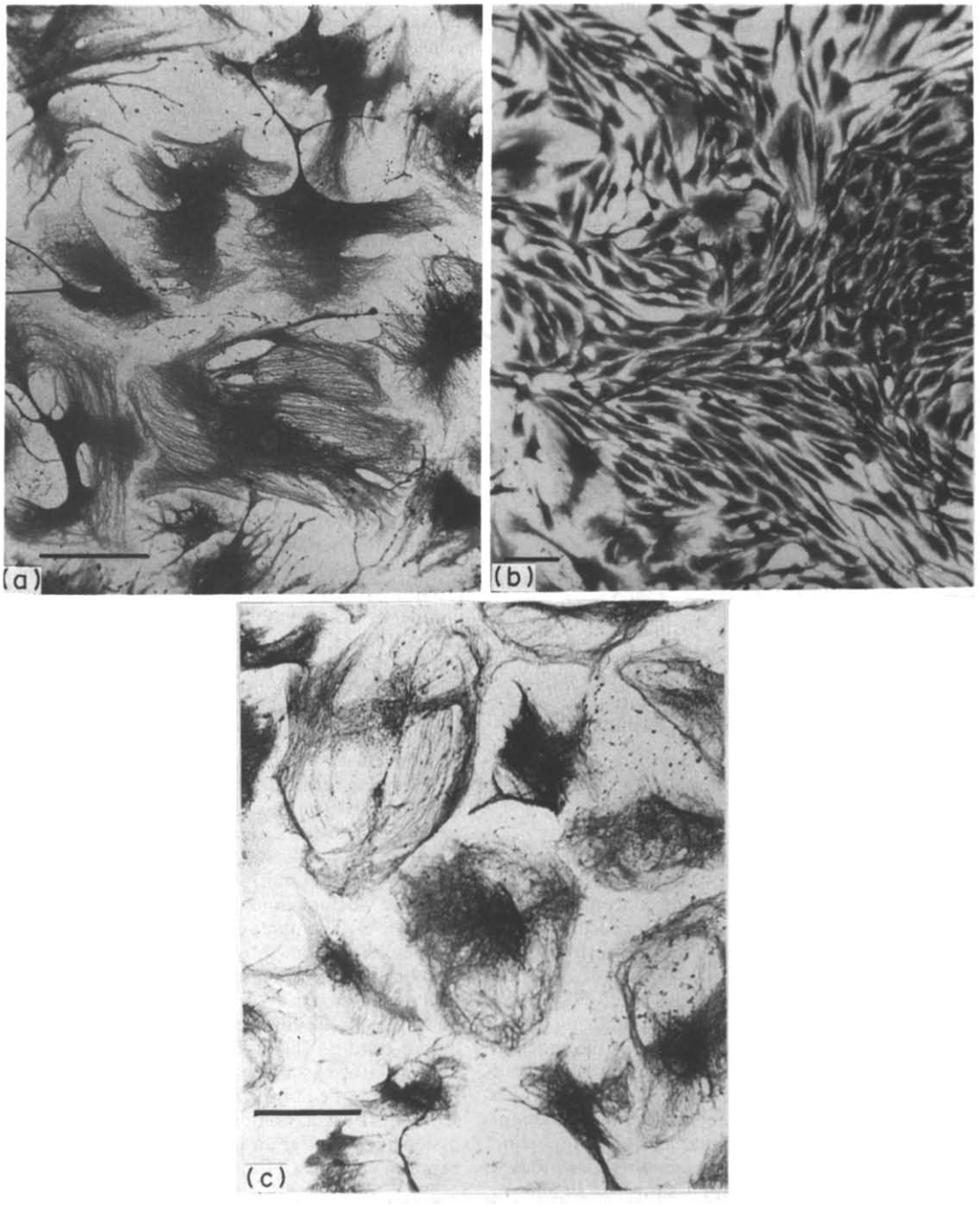

Fig. 4. Cytoskeleton staining of typical growing (a, c, e) and cluster forming (b, d, f) fat storing cells. Cells were incubated with 50 units rIFN-γ/mL as described in Table 3. At the seventh day of culture cells were stained for vimentin (a, b), desmin (c, d), and α-actin (e, f) as described in Materials and Methods. Bar = 0.1 mm.

noted in serum-supplemented cultures. Reasons for the diverse effects are unknown but a requirement for co-factors for the stimulatory action of rIFN-γ is suggested by the observation that recombinant IFN-γ shows smaller effects than the crude or purified cytokine, which was explained by contamination with traces of lymphotoxines [18]. In addition, the proliferation activity of fat storing cells is known to be sensitive to retinoids, of which the extracellular level is reduced under serum deprived conditions and, thus, might influence the action of rIFN. Although we cannot rule out a role of serum retinoids as a cofactor, fat storing cells isolated from vitamin A supplemented rats responded to the cytokine in a manner similar to that reported here (data not shown). Intracellular events related to the inhibitory effects of rIFN-γ on growth might be based on elevation of intracellular cAMP and cGMP levels

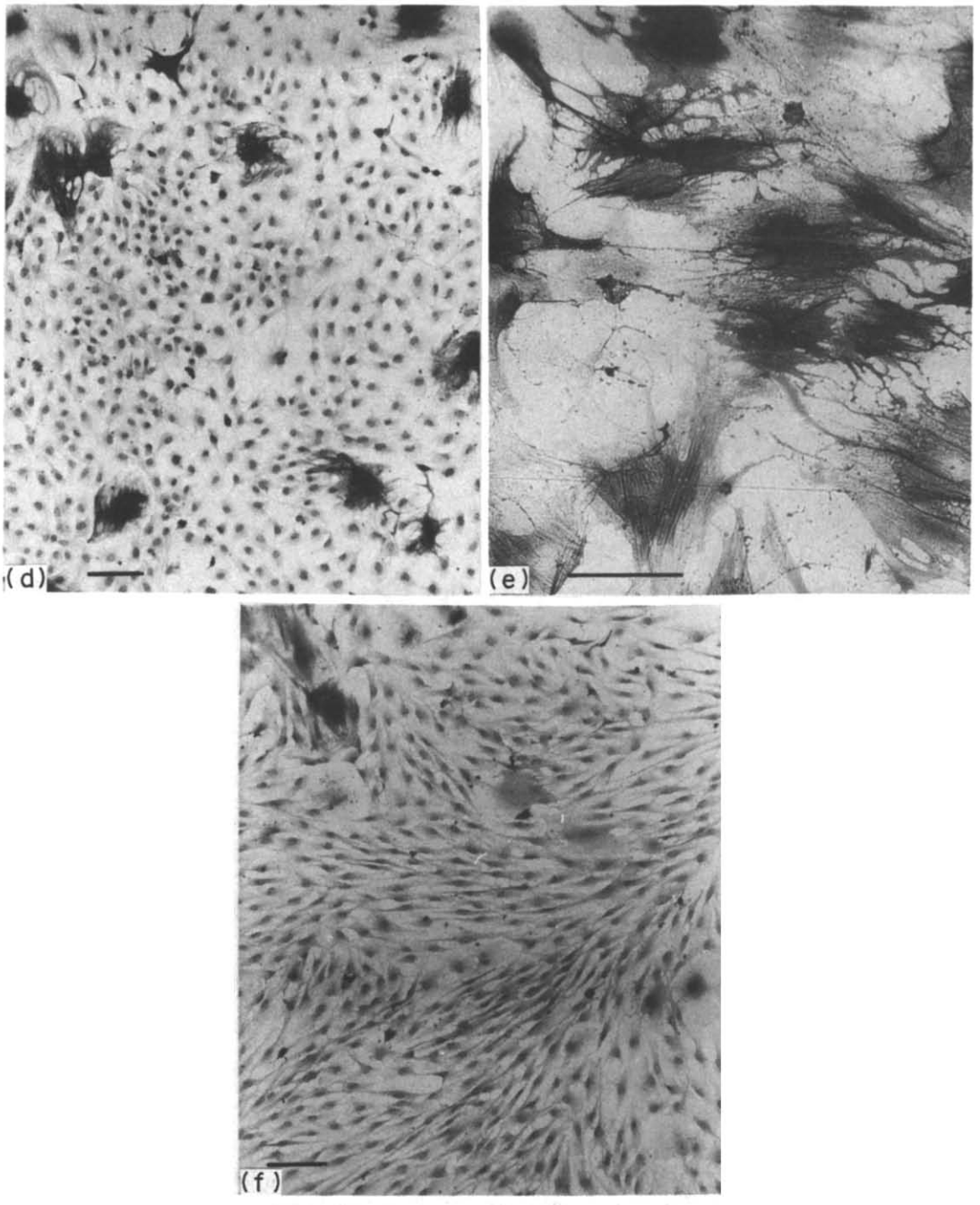

Fig. 4. *Continued.*

[12, 16], induction of 2′,5′oligoadenylate synthetase [45], effects on PGE_2 production [46] and on direct cytotoxic actions [12, 16]. Previous studies reported modulation of [^{3}H]thymidine uptake into cells by rIFN-γ [45, 47]. To rule out possible effects of rIFN-γ on membrane transport of thymidine and consequently significant changes of intracellular thymidine pool specific activity we studied the kinetics of uptake but did not find any significant differences between controls and IFN-γ treated cells. Furthermore, the use of hydroxyurea, an agent which inhibits selectively the replicative fraction of DNA synthesis [44] clearly demonstrates that the enhancing effect of rIFN-γ on [^{3}H]thymidine incorporation concerns the replicative but not reparative fraction of DNA synthesis. Taken together, the results provide evidence for a stimulation of proliferation of fat storing cells in calf serum supplemented medium by rIFN-γ.

The growth response of cultured fat storing cells to rIFN-γ was heterogeneous as observed by phase

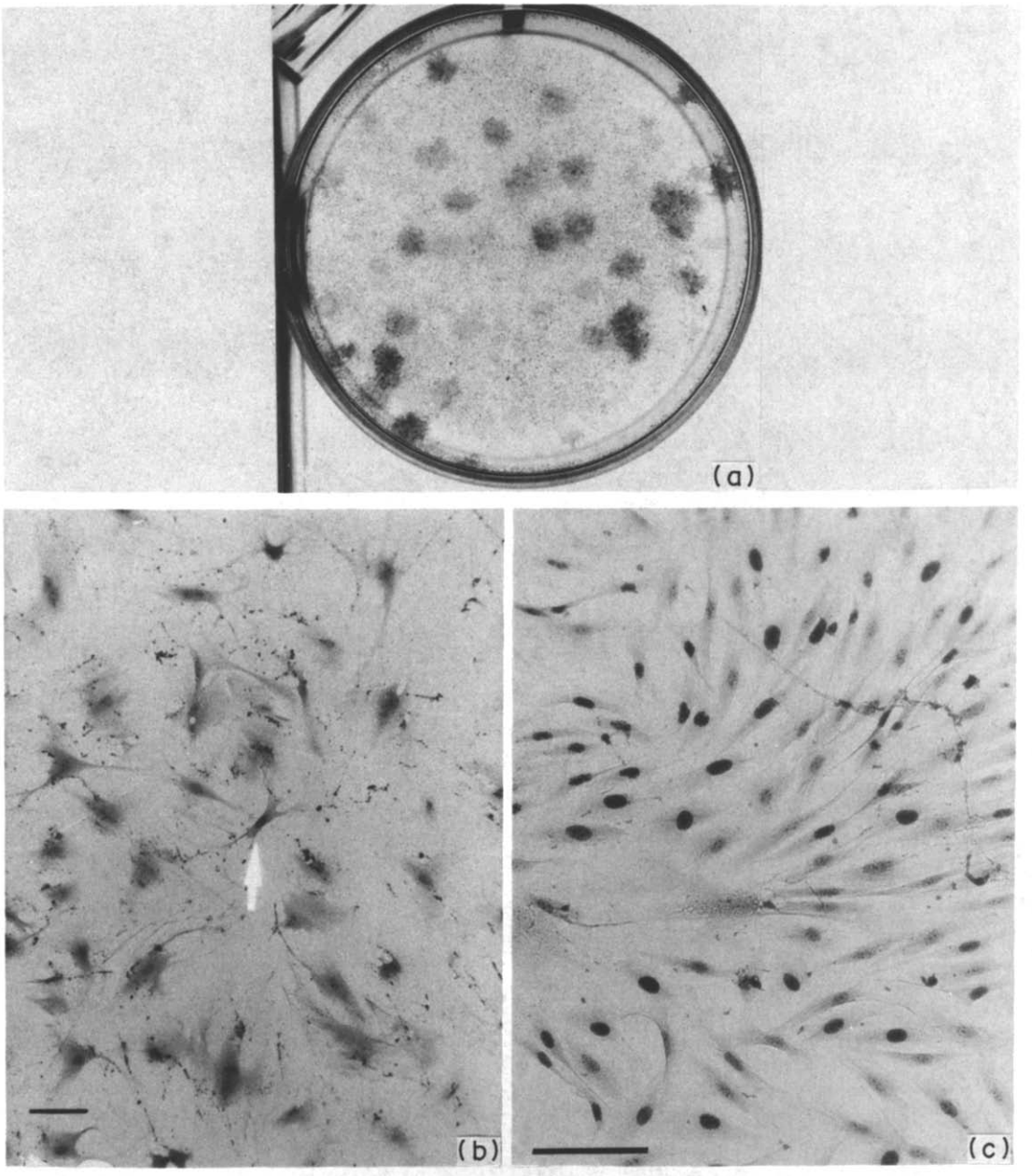

Fig. 5. Bromodeoxyuridin staining of fat storing cells at the eighth day of culture. Cells were treated with rIFN-γ (50 units/mL medium) as described in Table 3. Staining for BrdUrd was carried out as described in Materials and Methods. (a) Shows the positively stained clusters in a rIFN-γ treated culture (size of the culture well = 10 cm^2). (b) Microscopic picture of typically growing and (c) of cluster forming fat storing cells. Arrow in b = positively stained nucleus, bar = 0.1 mm.

contrast microscopy and even by macroscopic examination of late cultures. The lymphokine stimulated the time-dependent formation of cell clusters being in a highly proliferative state as evidenced by bromodeoxyuridine staining. This phenomenon poses the question about the nature of these clusters. The cells exhibited a typical morphology, which was distinct from that of the surroundings showing flat, mainly desmin-, α-actin- and vimentin-positive cells frequently laden with some lipid droplets and having cellular extensions. Thus, non-clustered cells were recognized as typical fat storing cells described previously by us [8] and others [2, 3]. Since the cluster forming cells were almost free of droplets, did not show appreciable vitamin A fluorescence, had elongated and smaller shape with a prominent nucleus and essentially did not stain with desmin and α-actin antibodies one might suspect that the clusters derive from a contaminating cell type. However, we regard this possibility as unlikely since careful

Table 3. Time-dependent effect of rIFN-γ on cluster formation in primary cultures of fat storing cells

Culture day	Number of clusters/culture well (10 cm^2)
3	3 ± 1.2
5	9 ± 1.9
6	13 ± 3.3
7	26 ± 3.5

Cells (seeding density $0.2 \times 10^5/cm^2$) were kept in medium containing 10% FCS. Medium was changed every 48 hr and new rIFN-γ (50 units/mL) was added. Cell clusters were identified and counted at the days indicated by visual examination of each four wells of three separate isolations using phase contrast microscopy and staining with crystal violet. Mean values ± SD are listed.

examination of early fat storing cell cultures after the second change of medium (first culture day) revealed a purity of about 98%, the contaminating cell types were identified to be mainly Kupffer cells and some endothelial cells. We did not observe fibroblasts or similar cell types. Therefore, we assume that the clusters are derived from a subpopulation of fat storing cells maybe by expressing a qualitatively and quantitatively different set of growth factor receptors and/or autocrine secretion of the respective ligands (growth factors) leading to self-stimulation [48]. Overexpression of ligand-activated receptors (e.g. EGF/TGFα, insulin) has been shown to be a condition for cellular transformation and cluster formation [48, 49].

Heterogeneity of fat storing cells has been reported before by showing subpopulations with different densities [50] and significant differences in the expression of desmin in culture [51]. Also we noticed a certain fraction of desmin and α-actin negative fat storing cells in late cultures. Our results support the notion [51] that desmin-staining cannot be used as a definite phenotypic marker of fat storing cells. Instead, the data suggest the presence of at least two subpopulations of fat storing cells being desmin positive and negative, respectively, and having different mitotic activities just as it was postulated by Ballardini *et al.* [51]. Growth factors and cytokines including rIFN-α might have different actions on the various subpopulations of fat storing cells and, therefore, may promote cluster formation.

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