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Overview: BLRs as Inducers of In Vivo Leucocyte and Platelet Genesis

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Zusammenfassung

Kurze Kette von Einzel-RNS-Fragmenten, sogenannte BLRs, durch sanften Abbau des aufbereiteten Escherichia coli ribosomale RNS mit Hilfe spezifischer Bauchspeicheldrüsenenzyme gewonnen, wirken als Auslöser für die in vitro Replikation der DNS, die aus Säugetierknochenmark und Milz isoliert wurde. Verabreicht man sie I.V. oder per os Kaninchen, die sowohl mit wie auch ohne Zytostatika behandelt wurden, sammeln sich die BLRs im Knochenmark und führen zu einer Vermehrung der Leukozyten und Thrombozyten ohne drohende Nebenwirkungen. Die BLRs, die einen Überschuß an Purin-Basen enthalten, zeigen relativ bedeutenden Widerstand gegenüber des Plasma-RNS-Abbauenzyms. Das BLR-Halbleben beträgt 8 Minuten. Magnesiumionen schützen die BLRs gegen diesen Abbau. Wiederholte Verabreichung von BLRs bewirkt kein Toleranzphänomen im Gegensatz zu bakterieller Endotoxin. Bei Säugetieren sind BLR nicht toxisch und führen nicht zur Krebszellenvermehrung. Ihre biologische Wirkung auf Leukozyten und Thrombozytenformation kann als Modell betrachtet werden, welches illustriert, wie eine kurze Kette von RNS-Molekülen selektiv eine Wirkung ausüben kann, sowohl auf die DNS-Aktivität als auch auf Zellenreproduktion, ohne irgendeine Nebenwirkung auf die physiologische Regulation von Säugetieren auszuüben.

Schlüsselwörter

DNS, RNS, RNS-Fragmente, Zytostatika, Leukozyten, Thrombozyten, Endotoxin.

Summary

Short chain single stranded RNA fragments, termed BLRs, obtained from mild degradation of purified *Escherichia coli* ribosomal RNAs with panreatic RNAse, act as primers in the *in vitro* replication of DNA isolated from mammalian bone marrow and spleen. When administered I.V. or per os to rabbits, treated or not with antimitotic drugs, BLRs concentrate in the bone marrow and induce an increase of leukocyte and platelet counts without impeding drug effect. BLRs, which contain an excess of purine bases, show a relatively important resistance to plasma RNAse. BLRs half-life is 8 minutes. Mg^{2+} ions protect BLRs against degradation by plasma RNAse. Repeated administration of BLRs does not induce any tolerance phenomenon contraty to bacterial endotoxin. In mammals, BLRs are not toxic and do not stimulate cancer cell multiplication. Their biological effect in leukopoiesis and platelet formation can be considered as a model illustrating how a given short chain RNA molecule can selectively exert its action both on the expression of gene activity and cell reproduction without disrupting mammalian physiological regulation.

Keywords

DNA, RNA, RNA-fragments, cytostatic, leukocytes, Thrombocytes, Endotoxin.

Introduction

Since 1970, it has been clearly established that under well defined conditions, the replication of single stranded DNA by DNA-dependent DNA polymerase, the first step of cell division, requires RNA primers in addition to all the necessary components for DNA synthesis [1-3]. In 1974, we established that a short RNA molecule can selectively exert its action on both the expression of gene activity and the reproduction of bone marrow cells, without disorganizing physiological regulation in animals. We demonstrated that specific short chain RNA fragments (obtained from the enzymic breakdown of *E. coli* purified ribosomal RNAs) acted as primers for *in vitro* replication of hemopoietic tissue DNA. The relative amount of their nucleotides (A,G,C,U) apparently determines the specificity of action of RNA fragments on hemopoietic cells [4].

We have previously described the biochemical and physiological properties of some specific RNA fragments termed BLRs. They are capable of inducing leukocyte and platelet count increase in rabbits when both types of blood cells have been decreased by various anticancer drugs. We also showed, with reverse transcriptase, that RNA fragments were not transcribed into DNA.

It was then of interest to describe the effect of BLRs, administered by the subcutaneous and oral routes, on leukocyte and platelet genesis in rabbits, untreated or treated with anticancer drugs, and to study BLRs *in vitro* degradation by animal plasma and the protective effect of Mg^{2+} ions.

Material and Methods

White New Zealand male rabbits were used for the experiments described here and elsewhere [5]. Purified ribosomal RNAs from *E. coli* were treated with pancreatic ribonuclease (have a phosphate activity which removed 3'-phosphatase from RNA fragments) to prepare particular RNA fragments termed. BLRs. Cyclophosphamide (Endoxan) was supplied by Laboratoire Lucien Colombes, France; it was always freshly prepared prior to injection. Purified endotoxin (LPS from *E. coli*) serotype 0111 B4 (Difco) was kindly provided by Dr. *M. Roumiantzeff*, Institut

Mérieux. A grade chemicals were used throughout. Poly A and Poly C were purchased from Miles Laboratory, USA.

Cyclophosphamide treatment
Rabbits (2,5-3 kg) were given daily intraveinous injections of 25-35 mg cyclophosphamide (CP)/kg. These amounts of CP induced a progressive decrease of circulating leukocyte (and platelet counts), which fell from 10^4 to roughly 5.0×10^3 cells / mm^3. When leukocyte count was decreased by about 50%, rabbits were regularly given cyclophosphamide and BLRs. A Coulter electronic cells counter was used to determine leukocyte and platelet counts. The proportion of granulocytes and lymphocytes was evaluated under the microscope (minimum 200 cells) after spreading and fixation of blood cells using the May-Grunwald Giemsa method.

Plasma collection
Blood from healthy rabbits was drawn from ear veins using EDTA anticoagulant, then centrifuged at 5,000 rpm to eliminate red blood cells. Plasma was used as such or after dilution (1/5) as a source of ribonuclease for BLRs degradation. ^{3}H-Guanine and ^{3}H-Uracil-labelled BLRs were prepared from labelled ribosomal RNA as previously described (5).

Degradation of ^{3}H-BLRs by plasma
The *in vitro* degradation of ^{3}H-BLRs at 36 °C was performed under conditions described elsewhere (5). Since BLRs are small (2-3 S), the reaction was stopped by an excess of ethanol (95 °C) + 5 µmoles of KCl in order to precipitate the ^{3}H-labelled insoluble material. Radioactivity was measured in a Packard liquid scintillation counter.
Since BLRs originate from *E. coli* ribosomal RNA, it was of interest to know the amount of endotoxin in our BLR preparations; it varied from 3 to 180 ng per mg of material tested. Poly (A), inactive in leukopoiesis, gave a positive reaction (162 ng/mg), while Poly (C) was poorly reactive (0.8 ng/mg). It must be underlined that various preparations (LAL, Limulus amaebocyte lysate) for endotoxin measurement contained different levels of RNAse that degraded BLRs (purine rich RNA fragments) and pyrimidine polymers, although the latter were only poorly degraded.

Results

DNA and protein-free BLRs were degraded *in vitro* by healthy rabbit plasma. Fig. 1 shows the degradation of ^{3}H-BLRs by healthy rabbit plasma (0.7 mg protein/assay) with time, at 36 °C (plasma protein content is 60-65 mg/ml). In the absence of Mg^{2+} ions, BLRs half-life in rabbit plasma is 8 minutes. It was calculated from the values in the linear portion of the curve (mean value for 3 different experiments). Degradation was linear (with undiluted or diluted plasma) for given plasma concentrations. In the presence of Mg^{2+} ions and under the same conditions, no degradation of ^{3}H-BLRs occured during the first three minutes of incubation and, afterwards, degradation was much lower than that observed in the absence of $MgCl_2$ (fig. 2). These data indicate that on the one hand BLRs are relatively resistent to plasma RNAse activity and on the other hand that Mg^{2+} acts as the BLRs protector.

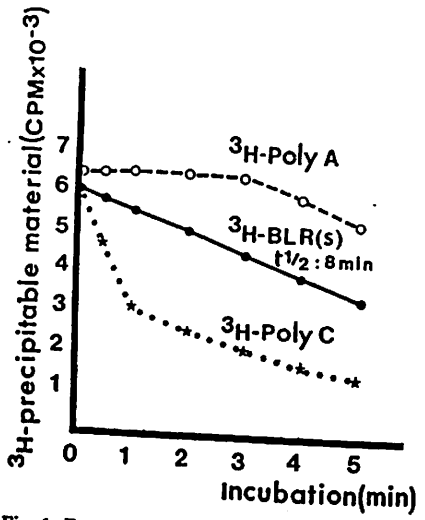

Fig. 1: For each time indicated in the figure, the incubation mixture (final volume 0.15 ml) contained 25 µmoles) of Tris-HCl buffer, pH 7.65; 200 µg of ^{3}H-BLRs (7,000 CPM); 0,01 ml of plasma (0,7 mg of protein). The same experiment was performed with ^{3}H-Poly A (200 µg, 7,200 CPM) and ^{3}H-Poly C (200 µg, 7,160 CPM). Incubation at 36 °C at times indicated on the figure. To stop the reaction, alcohol (95°) was added in excess oand then 5 µmoles of KCl to precipitate the ^{3}H-labelled insoluble material. The precipitate was filtered on a glass GF/C millipore filter, washed with ethanol, dried, and its radioactivity was measured with a Packard liquid scintillation counter (Prias). The results are expressed as CPM. The half-life (t 1/2 = 0,693) was calculated from values in the linear portion of the curves [5].

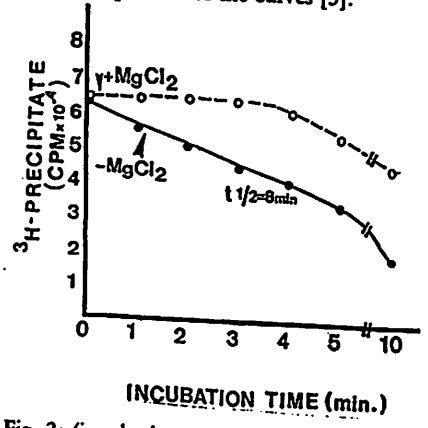

Fig. 2: (incubation conditions as in Fig. 1) Comparison between the degradation of ^{3}H-BLRs by healthy rabbit plasma in the absence and presence of $MgCl_2$ (1 µmole/assay). The results are expressed as CPM.

Oral administration of BLRs in endoxan pretreated-rabbits
The effect of orally administered BLRs on leukocyte and platelet counts (fig. 3) is slower and lasts longer than when BLRs are given by i.v. route [3]. Four rabbits were given daily 100 mg endoxan for 30 days and were injected BLRs i.v. to ensure survival. The administration of first BLRs and then endoxan was discontinued. The leukocytes counts were then 4,500-5,000/mm^3. After this treatment, rabbits cannot spontaneously restore leukocyte count within two weeks: a single dose of BLRs restores both leukocyte and platelet counts to normal within 10 days and these are maintained for a further 15 days approximately.

Leukopoietic activity of BLRs in methotrexate treated rabbits
Rabbits receiving daily, for 2 consecutive days, 60 mg I.V. or methotrexate, had a considerably reduced leukocyte count (fig. 4). They rapidly recovered a normal leukocyte count when BLRs were administered intraveinously, and slightly more slowly when they were administered subcunateously. After BLRs effect stopped, leucocyte counts decreased slowly.

Differential formation of granulocytes and platelets under the effect of various BLRs fractions
BLRs fractionation on a Sephadex G-25-fine column enabled us to identify fractions according to their

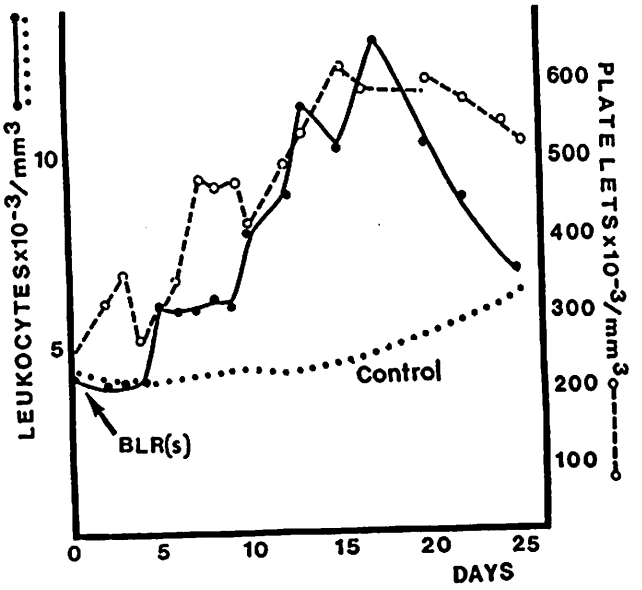

Fig. 3: This figure shows leukocyte and plasma counts increase in rabbits receiving 20 mg BLRs per os. Values represent an average of six rabbits. Initial leukocyte maximum count 4,500 ± 100/mm³ (a) increased up to 9,250 ± 2,200 ($p>0.02$) after BLR administration; initial platelet maximum count 250,000 ± 50,000 increased up to 575,000 ± 25,000/mm³ ($p>0.03$) after BLLR administration.
(a) values shown are arithmetic mean ± 1 S.D.

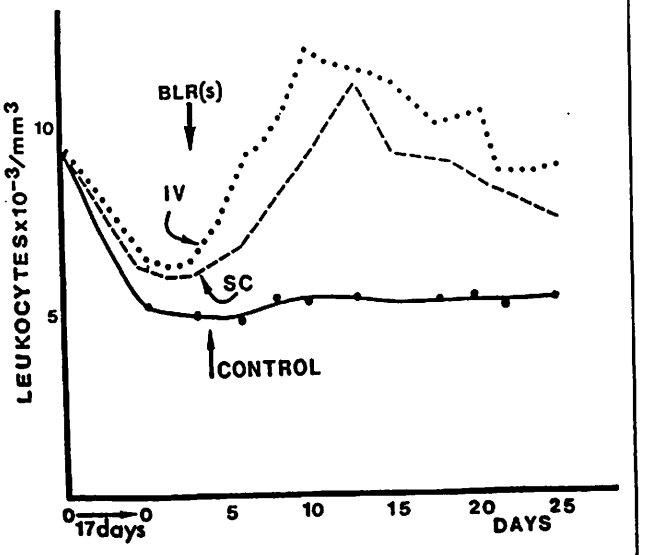

Fig. 4: Average values of leukocyte counts in methotrexate-treated rabbits (60 mg I.V. for 2 consecutive days), that received 5 mg of BLRs (as shown by arrows – I.V. = intraveinously; S.C. = subcutaneously). These values are typical of all rabbits treated with methotrexate [9].

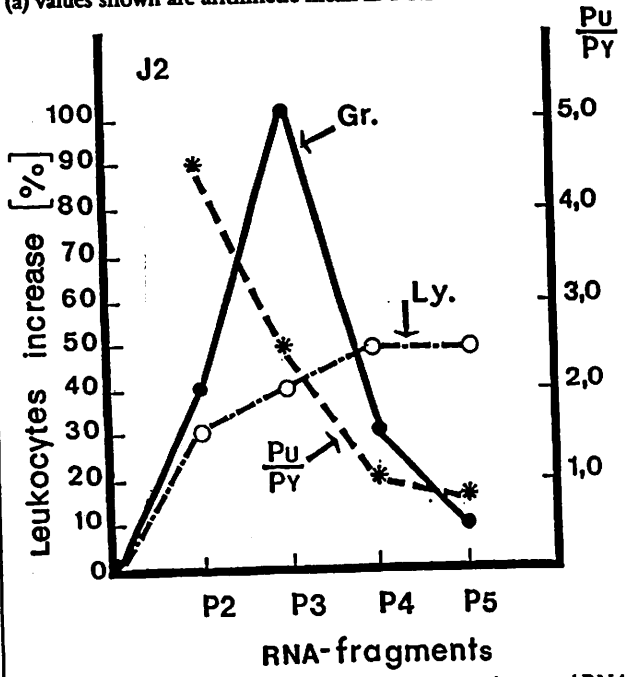

Fig. 5: BLRs prepared as described in the text contain several RNA fragments (P2, P3, P4, P5) which were separated on a Sephadex G-25 column [3]. The fractions have different purine/pyrimidine base ratios. They were tested in rabbits for lymphocyte and granulocyte formation. Each fraction (3-5 mg/rabbit) was injected I.V. to two healthy rabbits. At day two, blood was drawn and analysed for differential amounts of lymphocytes and granulocytes.

UV absorption at 260 nm (5 peaks were detected) [3]. The second eluted fraction, P2, had a base ratio (purine/pyrimidine) close to 5. The purine/pyrimidine ratio decreased in fractions eluted after this. Each one of these fractions was administered to two rabbits (3-5 mg per rabbit) by the intreaveinous route. On day 2, granulocyte and lymphocyte counts were determined. For granulocyte formation, the most active fraction (P3) had a base ratio of 2.5. This was also the average ratio for BLRs. In contrast, the most active fractions (P4 and P5) for lymphocyte formation had a base ratio close to 1.0. These results indicate that RNA fragments containing purines in excess act essentially as promoters of granulocytes, while those with a base ratio close to 1, act as promoters of lymphocytes, with a better yield than fractions P2 and P3 (fig. 5).

A comparison of E. coli endotoxin and BLRs effects on leukopoiesis in endoxan treated rabbits

Data mentioned above shows that BLRs physiologically induce a progressive increase in leukocyte count

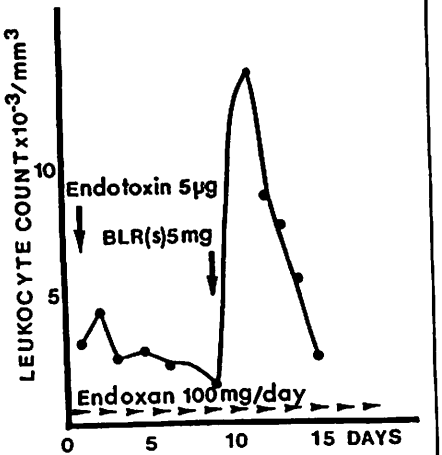

Fig. 6: Rabbits used exhibited tolerance phenomenon to endotoxin as described elsewhere [10]. Cyclophsophamide (endoxan) was injected (i.v.) daily (100 mg/day). Endotoxin was inactive while BLRs strongly increased leukocyte count.

which reaches its maximum level 48 h after administration [10]. Fig. 6 shows that 5 µg of bacterial endotoxin/kg administered to a rabbit receiving daily 100 mg of endoxan, produced a rapid increase of leukocytes (essentially granulocytes) during 16-24 h. Then, the leukocyte count went down because endoxan was administered every day. In contrast, peak of activity decreased rapidly, so that 48 h after LPS (lipopolysaccharide) administration the leukocyte number was practically equal to that observed just prior to injection.

BLRs administration (fig. 6), at indicated intervals, induced a progressive increase of leukocytes which reached its maximum level 48 h after administration. Then the leukocyte count decreased progressively but slowly. It took generally 5 days to fall back to initial level induced by CP treatment. The differential behaviours of endotoxin and BLRs clearly showed that BLRs did not act on the same target. In fact this difference was obvious from *in vitro* replication of bone marrow DNA in the absence or presence of either endotoxin or BLRs. Endotoxin had no stimulatory effect on bone marrow and spleen DNA *in vitro* replication, while BLRs were potent primers in this reaction (fig. 7).

It must be stressed that endotoxin did not induce an increase of platelet count while BLRs did so. Moreover, endotoxin induced a tolerance phenomenon after 3-5 successive administrations to animals, while nothing of the kind was observed with BLRs.

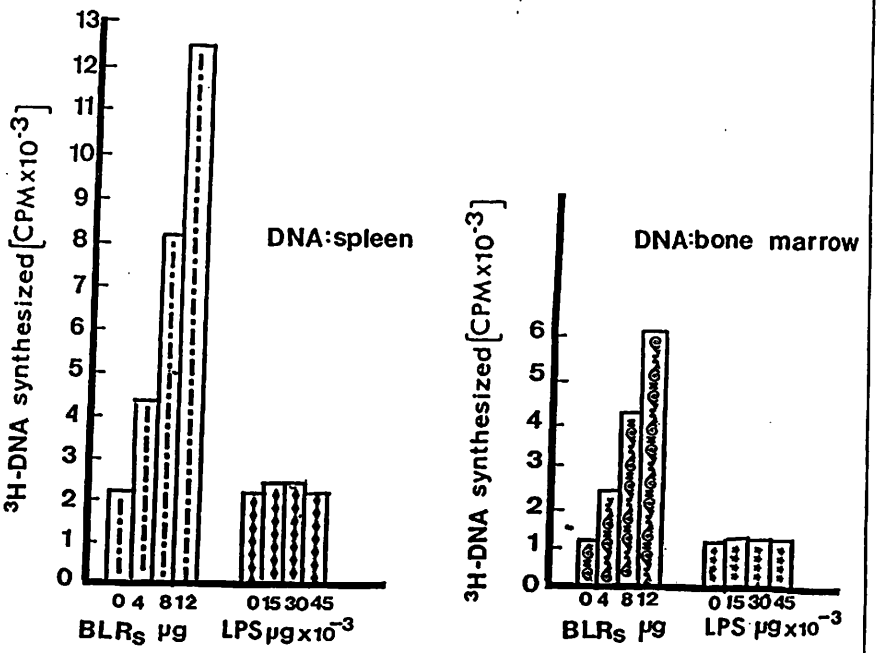

Fig. 7: ^{3}H-DNA synthesis was performed as decribed elsewhere [5]. BLR and endotoxin (LPS) concentrations are indicated on the figure.

Discussion and Comments

We described here how a specific short chain RNA molecule can selectively enhance leukocyte and platelet multiplication in animals. This *in vivo* activity of BLRs could be expected from their *in vitro* priming effect on the replication of DNA isolated from rabbit bone marrow and spleen. Moreover, *in vivo*, injected ^{3}H-BLRs localize in blood stem cell-containing tissues [6].

BLRs have been tested in rabbits treated with high doses of antimitotic drugs such as cyclophosphamide (endoxan) or methotrexate (amethopterin), which inflict severe injuries to the multiplying blood cell population because they are toxic to stem cells [5]. In these animals, which have become highly leukopenic and/or thrombocytopenic, BLRs promptly restore normal circulating leukocyte and platelet counts. BLRs contain components which differentially increase granulocyte and lymphocyte counts.

Many observations concur to show that BLR activity mimics physiological processes and fully integrates into cell life:

1. the increase of leukocyte and platelet counts produced by BLRs never exceeds normal physiological limits and there is no cumulative effect following repeated dosages;
2. an excess BLR dosage never produces overhigh counts of circulating leukocytes and platelets (this is certainly connected to the finite life span of these blood cells);
3. BLRs administered i.v. or per os increased platelet counts in both untreated and endoxan-treated rabbits;
4. a normal balance between polynuclears and lymphocytes is restored within a few hours following BLRs injection;
5. BLRs are not toxic;
6. BLRs do not induce a tolerance phenomenon as does endotoxin;
7. BLRs have no priming effect on the *in vitro* replication of DNA from various cancerous tissues and stimulate neither cancer cells in mice nor pathological leukocytes. Endotoxin does not act as primer for the *in vitro* bone marrow and spleen DNA replication.

We must add that BLRs are not toxic. After they have acted, they are degraded by endogenous endonucleases present in animal tissues.

Comparison with endotoxin shows that, contrary to this molecule, BLRs do not induce tolerance and their activity is not due to the release of leukocytes and platelets from a

reserve pool. In addition, endotoxin has no priming effect on bone marrow and spleen DNA replication. BLRs, for some time past, have been used for human therapy [6-8] where their properties have been fully confirmed, indicating that BLRs may contribute in a very positive way to the cure of leukocyte and platelet deficiencies, whether induced by chemotherapy (particularly cancer therapy) and radiotherapy or by various ailments, such as medullary aplasia.

Literatur

[1] *Sugino, A., Hirose, S., Okazaki, R.:* RNA-linked nascent DNA fragments in Escherichia coli. Proc. nat. Acad. Sci. USA 69 (1972) 1863-1867.

[2] *Plawecki, M., Beljanski, M.:* Synthèse in vitro d'un ARN utilisé comme amorceur pour la réplication de l'ADN. Compte-rendu Acad. Sci. Paris 278 D (1974) 1413-1416.

[3] *Beljanski, M., Beljanski, M., Plawecki, M., Bourgarel, P.:* ARN-fragments, amorceurs nécessaires à la réplication in vitro des ADN. Compte-rendu Acad. Sci. Paris 278 (1975) 1413-1416.

[4] *Beljanski, M.:* French patent n° 76 16875.

[5] *Beljanski, M., Plawecki, M., Bourgarel, P., Beljanski, M. S.:* Leukocyte Recovery with Short-chain RNA Fragments in Cyclophosphamide-treated Rabbits. Cancer Treatment Reports 67 (1983) 611-619.

[6] *Beljanski, M.:* Cancer Therapy: a New Approach. Deutsche Zeitschrift für Onkologie 22 (1990) 145-152.

[7] *Donadio, D., Lobro, R., Causse, J. E., Nawrocki, T., Beljanski, M.:* RNA-Fragments (RLB) and Tolerance of Cytostatic Treatments in Hematology: a Preliminary Study about two non-Hodgkin malignant Lymphoma cases. Deutsche Zeitschrift für Onkologie 23, 2 (1991) 33-35.

[8] *Grandi, M., Cras, N., De Kuyper, P.:* RNA-Fragments as Primer in the Disorders of Platelets and Lymphocytes: Preliminary Results. Second World Week of Professional Updating in Surgery and in Surgical and Onkological Disciplines of the University of Milan. 15-20 July 1990 (Ed. Monduzi).

[9] *Beljanski, M., Plawecki, M., Bourgarel, P., Beljanski, M. S.:* Short Chain RNA Fragments as Promoters of Leucocytes and Platelet Genesis in Animals depleted by Anticancer Drugs, In the Role of RNA in Development and Reproduction. Second Intern. Symposium, 25-30 April 1980, Beijing: 1981, Science Press (M. C. Niu, H. H. Chuang eds), pp. 79-113.

[10] *Plawecki, M., Beljanski, M.:* Comparative Study of Escherichia coli Endotoxin, Hydrocortisone and Beljanski Leukocyte Restorers Activity in Cyclophosphamide-treated Rabbits. Proc. of the Soc. for Exp. Biol. Med., 168 (1981), pp. 408-413.

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Naturheilkunde bald Lehrfach an deutschen Unis — Verlag für Medizin Dr. Ewald Fischer wird 25

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Diese Entwicklung ist nicht zuletzt ein Verdienst des Heidelberger **Verlages für Medizin Dr. Ewald Fischer** — kurz **vfm**. Durch die Gründung des **Verlages für Medizin** vor nunmehr genau 25 Jahren wollte der Verleger Dr. *Ewald Fischer* eine Brücke schlagen zwischen traditioneller Schulmedizin und naturheilkundlichen Verfahren. Ziel ist eine einheitliche Medizin, die nicht unterteilt wird in eine offizielle und eine alternative Medizin und die somit alle Möglichkeiten zur Heilung ausschöpfen kann.

Zusammen mit dem traditionsreichen **Karl F. Haug** Verlag, der bereits seit 1903 Bücher über Homöopathie, seit 1950 Bücher und Zeitschriften über Akupunktur und Neuraltherapie veröffentlicht, bildet **vfm** die Verlagsgruppe der Heidelberger **Medizin-Verlage.**

Das schulmedizinische Buchprogramm von **vfm**, der ursprünglich Verlag für Physikalische Medizin hieß, umfaßt über 160 lieferbare Titel aus den Gebieten Onkologie, Immunologie, Bewegungstherapie, Spurenelemente und Manuelle Medizin. Zu den **vfm**-Autoren gehören bekannte Heidelberger Mediziner wie Prof. Dr. *Hans Schaefer* („Medizinische Ethik", „Das Prinzip Psychosomatik") und Prof Dr. *Heinrich Schipperges* („Der Garten der Gesundheit", „Die Sprache der Medizin").

Eine Reihe von Patientenzeitschriften beschäftigt sich regelmäßig mit Volkskrankheiten wie Rheuma, Krebs, Herz- und Kreislauferkrankungen sowie mit Neurodermitis. Die Zeitschriften „Mit Rheuma leben", „Signal – Leben mit Krebs", „Hautfreund" (Zeitschrift des deutschen Neurodermitikerbundes) und der „Paraplegiker" (Das Nachrichtenmagazin für Querschnittgelähmte) geben dem Betroffenen die Möglichkeit, sich umfassend zu informieren und selbstbestimmt mit ihrer Krankheit umzugehen.