Regular Paper ISSN- 0102-9010

BIREFRINGENCE OF CELLS GROWN *IN VITRO* AND OF MITOTIC CHROMOSOMES AFTER STAINING WITH PICROSIRIUS RED*

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ABSTRACT

Fibroblasts and neuroblastoma cells kept in monolayer cultures, as well as surface spreads of mitotic chromosomes, were stained with picrosirius red. Red staining (in normal light) and optical anisotropy of the stained structures (in polarized light) were observed intracellularly and in the chromosomes. The intracellular and extranuclear birefringence induced by staining with sirius red could not be abolished by digestion with collagenase prior to staining, or by treatments used to disrupt microtubules (vinblastine, colcemid) or microfilaments (cytochalasin B). We therefore propose that the parallelly-arranged intermediate filaments are responsible for the optical anisotropy induced by sirius red staining in these cells. In addition, the spatially oriented scaffold of chromosomes can be detected by sirius red-induced birefringence. These data argue against the collagen-specificity of picrosirius red staining and of the birefringence induced by this technique. Our results also suggest that picrosirius red staining combined with polarized light microscopy can be used to study the spatial orientation pattern of the intermediate filaments and chromosome scaffold.

Keywords: Birefringence, chromosome scaffold, cytoskeleton, polarized light microscopy, picrosirius red

INTRODUCTION

Polarized light microscopy is a classic and still useful technique for studying the ultrastructure of different biological structures. The potential of polarized light microscopy is greatly enhanced by combining staining and histochemical reactions. Certain labeling techniques enhance the optical anisotropy of a variety of cellular or extracellular structures, including macromolecules in a selective manner. If the specificity of the labeling reaction is ensured, a qualitative or quantitative analysis of the amplified optical signal, i.e. the optical anisotropy, seen in polarized light microscopy may provide evidence about the spatial orientation pattern of the labeled structure at the molecular level. The pioneering studies of this research technique include those of Schmidt [19], Romhányi [15,16], Vidal [24,25,28], Mello and Vidal [11], Scheuner and Hutschenreiter [17] and Módis [12]. One set of special histochemical labeling methods has been proposed for amplification of the optical anisotropy of collagen. One of the most widely used reactions in collagen research is picrosirius red (PSR) staining combined with polarized light microscopy. The significance of this technique has also been demonstrated by quantitative data of Vidal *et al*. showing 5-6 times increase of optical retardation values of the collagen bundles after staining with PSR [27].

Originally, the sirius red (F3B or F3BA), an anionic dye, was proposed to replace acid fuchsin in the van Gieson procedure to obtain a more stable red staining of collagen [22]. Constantine and Mowry [2] observed that the optical anisotropy of the collagen was considerably enhanced after staining with PSR (0.1% sirius red dissolved in a saturated solution of picric acid in water). An overview of sirius red binding and its use as a topo-optical histochemical reagent has been provided by Módis [12]. Unfortunately, there is considerable inconsistency in the terminology used and this had led to some confusion in the literature. Thus, some authors refer to sirius red

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* This paper is dedicated to Professor Benedicto de Campos Vidal on the occasion of his 75th birthday.

alone when they actually mean PSR staining. Others describe PSR staining as collagen-specific [12], or even collagen-type specific [1]. Some authors restrict this definition to birefringent collagen bundles only [1,2], while others use it in a general sense, regardless of the occurrence of optical anisotropy in the tissue sections stained [10]. Extreme cases in the use of this dye include determination of the collagen content by colorimetric quantitation of the amount of dye extracted from material stained with sirius red [29].

In the present paper, in agreement with the caveat put forward by Módis [12], we describe examples that strongly question the collagen-specificity of PSR staining and birefringence.

MATERIAL AND METHODS

Adherence-dependent mouse fibroblasts (L_{929}) and neuroblastoma cells (NB_2A) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 5% fetal calf serum (Sigma), on precleaned, sterile coverslips, using standard cell culture techniques. Occasionally, neuroblastoma cells were deprived of serum until they developed more pronounced, axon-like processes. The organization of microtubules and microfilaments in the cytoskeletal network of fibroblasts was disrupted with vinblastine (Richter Pharmaceutical Works, Budapest, Hungary) or colcemid (a generous gift of CIBA-Geigy, Basle, Switzerland) added to the culture medium at final concentrations of 0.5 μg/ml to 10 μg/ml, and with cytochalasin B (0.1 to 20 μg/ml; Sigma), respectively. Duration of these treatments ranged from 60 min to 16 h. At 30-40% confluence, the glass coverslips with the cells were removed from the medium, rinsed twice in phosphate-buffered saline (PBS) and fixed in Kahle solution (60 ml of distilled H_2O, 28 ml of 96% ethanol, 10 ml of neutralized, concentrated formaldehyde, and 2 ml of glacial acetic acid). In some cases, the cells were extracted with a methanol:chloroform mixture (2:1,V/V), at 56°C for 105 min to remove any lipids remnant. After fixation, the coverslip cultures were rinsed in PBS.

Any collagen deposition by the cells was removed by treatment with collagenase (from *Clostridium histolyticum*, 0.65 U/mg, Serva, Heidelberg, Germany) at 2 mg/ml in 0.05 M Tris-HCl buffer, containing 1 mM $CaCl_2$, at pH 7.5, incubated at 37°C for 15 min to 8 h.

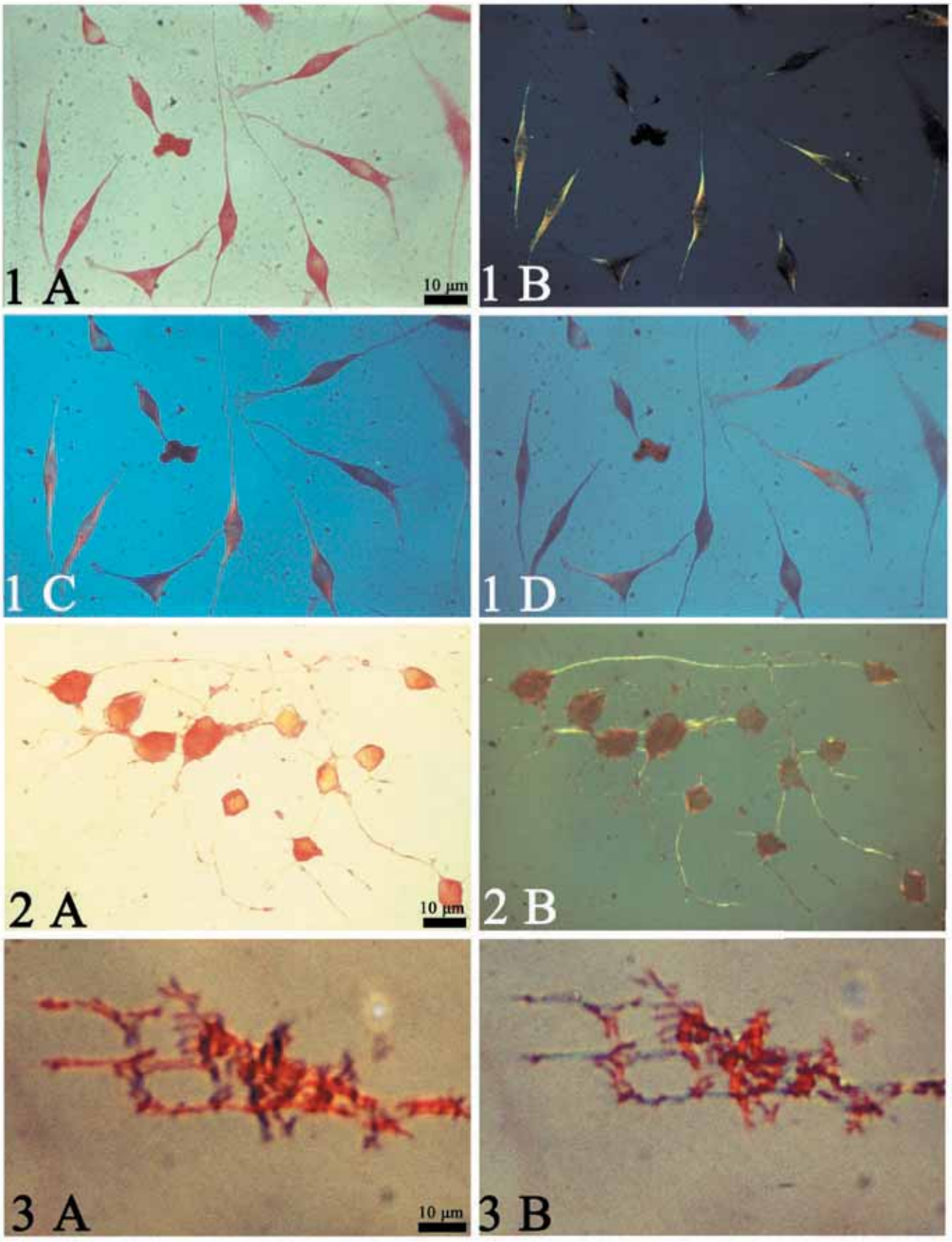
Mitotic chromosomes were prepared from mouse F4N erythroleukemia cells grown in suspension in RPMI 1640 medium (Sigma), supplemented with 10% fetal calf serum. The cells were lysed using the surface lysis method developed by Schlammadinger [18]. Briefly, cells arrested with colcemid (Gibco, USA) were suspended in PBS and surface spreads in 20 μl droplets were transferred to glass coverslips.

Cells and mitotic chromosomes were stained in 0.1% sirius red F3B (Bayer, Germany) dissolved in a saturated solution of picric acid in water (referred to here as PSR) for 30 min, according to Constantine and Mowry [2]. After rinsing and rapid dehydration in three changes of absolute ethanol, the samples were cleared in a 1:1 (V/V) mixture of ethanol:xylene followed by 3x3 min immersion in xylene, and the coverslips were mounted on standard slides with DPX.

Figure 1. Mouse L_{929} fibroblasts cultured on glass coverslips, and fixed and stained with PSR as described in the Material and Methods section. The same microscopic field is shown in each of the four images. **A:** Normal light. The nuclei are less intensely stained than the cytoplasm. Round (presumably mitotic) cells show intense coloration. **B:** Polarized light, between crossed polars. Note the intensely birefringent cytoplasmic bundles that are arranged predominantly parallel to the long axis of the cells and processes. Different colors indicate different thickness of the various biological structures. Neither the nuclei nor the cytoplasm of the round cells show any anisotropy. **C:** Additive compensation. A λ/10 Brace-Köhler compensator was placed in the light path so as to enhance the anisotropy of structures aligned in a north-south direction. This arrangement resulted in a orange-yellowish appearance, while the anisotropic parts of structures oriented in an east-west direction were blue as the result of a subtractive compensation. **D:** After turning the compensator in the opposite direction, the anisotropy of structures oriented west-east was enhanced and resulted in a yellowish color, whereas the anisotropy of structures in the north-south direction diminished, as indicated by the blue color (= subtractive compensation). The optical effect seen after the compensations clearly showed that the intracellular filaments bound the sirius red molecules in an axiparallel manner.

Figure 2. Serum-starved neuroblastoma cells that developed long, axon-like processes. **A:** Normal light. **B:** The same field in polarized light, between crossed polars. Moderate anisotropy can be seen in the round cell bodies. Axon-like processes show intense birefringence.

Figure 3. A group of mitotic chromosomes from colcemid-treated, isotonically lysed F4N cells, prepared as described in the Material and Methods section. In this case, the west-east direction was selected for additive (**A**) or subtractive (**B**) compensation, and resulted in the orange-red or blue colors of optical anisotropy, respectively. Mitotic chromosomes obtained from the liquid-air interface tended to be elongated because of the surface tension that generated stretching, and often aggregated, occasionally side-by-side. The latter behavior resulted in amplification of the chromatids that were oriented in parallel, thereby enhancing the effect on anisotropy. Birefringence was also visible in sister chromatids of obviously single chromosomes.


1 A
10 µm
1 B
1 C
1 D
2 A
10 µm
2 B
3 A
10 µm
3 B

The stained material was examined with a Reichert Zetopan-Pol microscope equipped with a λ/10 Brace-Köhler mica-compensator and a 100 W halogen lamp.

RESULTS

In normal light, a rather strong red coloration was observed in the cytoplasm (including cytoplasmic processes) of cultured cells after PSR staining. The nuclei were generally less apparent (Fig. 1A-D and Fig. 2A,B). In some cases, nucleoli were clearly visible. The staining was not abolished by digestion with collagenase, or by treatment with vinblastine or cytochalasin B. Occasionaly, the cytoplasmic staining was rather diffuse, with an evenly distributed coloration, while in other instances some fibrous structures were seen extending from the cell body into the processes.

In linearly polarized light, when the cells were viewed through crossed polars, the cytoplasm showed induced optical anisotropy that appeared in green, yellow and orange polarization colors (Figs. 1B and 2B). The sign of birefringence was always positive with respect either to the long axis of elongated cell bodies, cell processes, intercellular bridges and intracytoplasmic fibers (Fig. 1C,D), or to the tangent of circular structures (e.g. perinuclear baskets).

In neuroblastoma cells, the birefringence of long, axon-like processes was apparent (Fig. 2B). Little or no birefringence was seen in unstained cells mounted in PBS, gum arabic, or DPX.

In L_{929} fibroblasts, the cell processes were always filled with a birefringent material after PSR staining (Fig. 1B). The birefringence highlighted even the fine processes. The intensity of the cell body anisotropy depended on the shape of the cell. In long and short spindle-shaped cells, the birefringent structure was filamentous and oriented parallel to the long axis of the cell. No anisotropy was seen in mitotic spindles or in the ruffled borders of lamellipodia.

The birefringence was not abolished by treatment with collagenase (provided the cells remained attached to the substratum), or by treatment with vinblastine or cytochalasin B prior to fixation and staining.

The sign of anisotropy was also positive with respect to the longitudinal axis of the chromosomal bundles, indicating a similar orientation of the dye molecules in the case of isolated mitotic chromosomes (Fig. 3A,B). These morphological entities were quite well stained in some vinblastine-treated fibroblasts that were round but still more or less flattened and presumably in the prophase of mitosis. The chromosomes of these cells showed no anisotropy *in situ*.

DISCUSSION

In the last 35 years, sirius red staining has gained a rather wide acceptance in histology and histochemistry, although there is still considerable uncertainty in the use of sirius red terminology and in the specificity of the dye. Sirius red staining is often mentioned as being equivalent to the PSR method, especially in the abstracts of papers available from internet databases. Even when the original full paper is consulted, it can be very difficult to determine from the description of the methods, or from the cited literature, whether the authors used saturated picric acid solution as the solvent for the dye or some other medium.

The specificity of the staining is even more critical. In most papers, the birefringence seen after PSR staining of different tissues is considered to be collagen-specific. Almost 15 years ago, Módis [12] discussed the inappropriate nature of this assumption, which extends to the recently suggested collagen type I specificity of PSR birefringence [1]. In addition, the reliability of quantifying collagen in tissue samples stained with PSR, such as by image analysis techniques [4,21], by measurement under circularly polarized light [23] and by extraction and colorimetric determination of the dye [29] can be biased and misleading.

Although only a few publications have questioned the collagen specificity of sirius red, the studies involved were well done and should be taken in consideration by investigators using sirius red staining. Piérard [14] concluded that, “The sirius red polarization method is useful to visualize the organization of connective tissues and not to identify the molecular nature of their fibrous polymers.” Wehrend *et al*. [30] moved beyond connective tissue when they demonstrated that sirius red selectively stained the granules in eosinophil granulocytes. Nielsen *et al*. [13] pointed out that: “...acid fuchsin and Sirius red are not selectively bound to collagen; they are also bound to other proteins containing basic amino acids, and staining to a large extent is influenced by electrostatic forces.” Sirius red molecules can also bind to amyloid fibrils in an oriented manner [3].

Our findings reported here are in good agreement with the views of the last three studies cited above. We succeeded in staining cultured cells strongly with PSR. Under polarized light, this staining made the cells birefringent and revealed longitudinally arranged fibrous material within the cytoplasm. Assuming a parallel binding of the linear sirius red molecules to chromotropes, this optical finding indicates a predominantly parallel orientation of the stained components relative to the axis mentioned. Nuclei and nucleoli (as well as chromosomes within cells) were always isotropic. It is very improbable that the intracellular birefringent structures observed could be an organized, oriented deposition of collagen fibers since the supramolecular organization of the latter molecules occurs extracellularly. In agreement with this, treatment with collagenase did not abolish the staining or the anisotropy of the cells.

What type of organized, regular and unidirectionally oriented structures can be present in these and other cells? The obvious answer to this question is the cytoskeleton, which consists of three types of fibrillar-filamentous structures: microtubules (MT), microfilaments (actin filaments, MF) and intermediate filaments (IF), none of which can be excluded *a priori* a good candidate for induced birefringence. However, we suggest that the IF are the likely target since the dynamic and rapidly degradable polymerization of the two structures can be inhibited by vinblastine and colcemid or cytochalasin B, respectively. None of these inhibitors abolished the staining and birefringence in the cells studied. More convincing evidence for the involvement of IF would be provided by the selective destruction of this component of the cytoskeleton. However, the use of acrylamide provided no conclusive evidence for the involvement of IF since acrylamide-treated cells became tiny spheres in which it was impossible to visualize their ordered intracellular structures.

Our hypothesis is supported by an observation by Junqueira *et al*. [5] who described a mass of oriented, strongly birefringent intracellular structures in basal epidermal cells of tadpoles of the anuran *Pseudis paradoxus* stained with PSR. In addition, ample bundles of intermediate filaments arranged in a parallel distribution were detected by electron microscopy. From these observations, we conclude that the birefringent structures corresponded to IFs.

The birefringence of tonofilaments in rat skin treated with peanut agglutinin was described by Vidal and Carvalho [27]. More interestingly, these authors unexpectedly found strong birefringence in cultured, human breast epithelial cells not stained with sirius red and attributed this phenomenon to cytoplasmic tonofilaments. In the specimens examined by these authors the intermediate filaments, presumably tonofilaments, should have been present at high density and in a dominantly parallel arrangement.

The sirius red molecules alone are not responsible for the axiparallel molecular organization seen as a birefringent structure. Rather, they enhance the already existing but hardly visible anisotropy of the ordered biological structure. This enhancing effect is referred to traditionally as a topo-optical reaction [12]. The rather long sirius red molecules may attach longitudinally to the biological polymers, presumably by electrostatic interactions [12,13].

Mitotic chromosomes are highly and precisely organized structures formed from the chromatin material in eukaryotic cells [9]. As early as the 1930s, this high level of molecular order attracted the interest of polarized light microscopists who showed birefringence in plant [7] and polytene chromosomes [20]. Later investigators restricted themselves to studies on unstained dinoflagellate and dipteran polytene chromosomes, which showed positive and negative birefringence [8]. The birefringence of toluidine blue-stained mitotic chromosomes in mammalian cells was first demonstrated by Kellermeyer *et al*. [6] and Vidal [26]. To our knowledge, the anisotropy of unstained mammalian mitotic chromosomes was not observed. Similarly, there has been no report on the staining of mammalian mitotic chromosomes with sirius red as means of enhancing the anisotropy supposedly inherent in the highly sophisticated molecular order of these structures.

Sirius red molecules presumably bind to mitotic chromosomes released from cells by isotonic lysis [18] by the same mechanism seen in the cytoplasm of whole cells. The mitotic chromosomes undergo some stretching when the cells hit the air-water interface and are lysed. Whether the dye molecules bind to the chromosome scaffold or to histones [13] remains unclear. The chromosome scaffold is a corkscrew-like structure [9] that may assume a linear appearance upon stretching. If one assumes a similar binding of sirius red molecules to the expanded

(elongated) axis of chromosomes, as in the case of collagen fibers, then it is possible to understand that chromosomes show the observed birefringence presumably because of their side by side (parallel) formation, that leads to strand amplification. In mitotic figures obtained by the isotonic lysis of cells, the chromosomes prefer to adhere to each other [18]. The possible binding of sirius red to histone molecules [13] in adjacent chromatin loops, where these loops are extremely highly ordered, cannot be overlooked. In this case, the dye molecules would not be bound to the scaffold, but would be oriented by the scaffold upon binding to histone within mitotic chromosomes. However, this possibility seems less probable since the lateral parts of chromatids, which contain a similar amount of histone but have a considerably lower degree of oriented organization, could mask the ordered and axiparallely oriented binding of sirius red molecules to the scaffold attachment sites of the chromatin loops.

In conclusion, the results of these experiments are in good agreement with the findings of others, and argue against the widely accepted concept that the sirius red-induced birefringence is collagen-specific. Our findings also show that PSR staining combined with polarized light microscopy can be a simple, useful tool for studying the spatial orientation of the intermediate filaments and chromosome scaffold.

ACKNOWLEDGMENTS

The expert technical assistance of Ms. Ilona Oláh and Ms. Enikö Kovács is gratefully acknowledged. Dr. Szabolcs Felszeghy and Mr. Zoltán Király helped with the image digitization and editing. F4N cells were a generous gift from Dr. Donna Arndt-Jovin (Göttingen), L_{929} mouse fibroblasts were from Dr. Imre Mécs (Szeged) and NB_2A cells were kindly donated by Prof. Denis Monard (Basel).

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Received: January 5, 2005
Accepted: May 11, 2005