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# THE IMMUNOFLUORESCENCE VISUALIZATION OF ALR (AUGMENTER OF LIVER REGENERATION) REVEALS ITS PRESENCE IN PLATELETS AND MALE GERM CELLS

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# ABSTRACT

Utilizing sequence information from the recently cloned rat ALR (Augmenter of Liver Regeneration) cDNA, ALR peptides have been synthesized, and antibodies against ALR produced, which permitted the analysis of the sites of ALR expression by immunofluorescence. We found that ALR is present in platelets in the blood in the liver, the spleen, and indeed in blood vessels throughout the body. The tendency of platelets to be present in especially high quantity in the liver and the red pulp of the spleen would appear to account for why ALR protein and mRNA is so prominent at these sites. In the testes, ALR is present in the germ cells, including the spermatozoa, where it is present on the sperm tails.

# INTRODUCTION

The recent cloning of the rat, mouse, and human cDNA's and genes of the hepatotropic factor ALR (Augmenter of Liver Regeneration) has revealed that, in addition to its activity in maintaining liver integrity when administered *in vivo*, ALR also plays a quite distinct role *within* the cells in which it is produced (Fancavilla et al 1994, Hagiya et al 1994, Giorda et al 1996). Three findings pointed to the nature of this intracellular role: ***First***, from the sequence of the rat ALR cDNA, it became apparent that ALR is homologous to a yeast protein, ERV1, which plays an essential role in the control of mitochondrial gene expression (Lisowsky, 1992, 1994, Lisowsky, et al 1995). ***Second***, by Northern blot analysis ALR mRNA was found to be present in a wide variety of tissues, with strongest expression in the testes (Hagiya et al 199, Giorda et al 1996). ***Third***, the mouse ALR gene was found to map to the *T/t* complex of chromosome 17 of the mouse, a region long recognized for its influence on sperm function (Giorda et al 1996). Here we present immunofluorescence images, which reveal that ALR protein is expressed in platelets throughout the body, and in male germ cells in the testes. These findings raise the question of whether ALR plays a role in control of mitochondrial gene expression in platelets and male germ cells. These findings also raise the question of whether some of the phenotypic effects of *t*-mutations on sperm function could be accounted for by mutations of the ALR gene.

# MATERIALS AND METHODS

## Rats and Mice.

Female rats were were purchased from The Charles River Laboratory. Random bred male mice were kindly supplied by Dr. Mark Fishman.

## Immunofluorescence.

Mice and rats were sacrificed by $CO_2$ inhalation. In several experiments, the liver was perfused by passing phosphate buffered saline (PBS) into the left ventricle of the heart for approximately 3 minutes, or until the majority of blood was removed from the liver, turning it from a dark crimson to a beige color (10-15ml for mice). The perfused liver was then frozen on a block of aluminum on a bed of dry ice. Livers were sectioned on a Reichert-Jung 2800 Frigocut E at -25C at a thickness of four microns. The sections were then fixed by a 15 minute immersion in acetone, followed by four washes in PBS, five minutes each. Two-color immunofluorescence was then performed.

Typically, a mixture of rabbit and goat antisera in PBS was added to each section, usually rabbit anti ALR (#17Ig or #18Ig in most experiments (see below for definition, dilution 1/1), and goat anti-rat transferrin (Nordic Immunological Laboratories, Capistrano Beach, CA, Cat. Code GARa/Tfn, lot 42-285)or goat anti-mouse albumin (Nordic Immunological Laboratories, Capistrano Beach, CAD, Cat. Code RAM/Alb, lot 14-787); sheep anti-mouse albumin (Cappel Research Products, Durham, NC, Cat. #0111-0344, lot 26599). TRITC-conjugated swine anti-rabbit antiserum and FITC-conjugated donkey anti-goat IgG antiserum were mixed together in a 1/30 dilution in PBS. (Alternatively, visualization of the macrophage marker f4/80 on mouse liver was performed with rat-anti-mouse f4/80 (at 1/30 dilution) as the primary antiserum, and goat-anti-rat antiserum (at 1/30 dilution in PBS) as the secondary antiserum; sections were incubated at room temperature in a damp chamber for 45 minutes, then washed five times in PBS, five minutes each.) Each section was covered with 15 microliters of this solution and left at room temperature for 45 minutes in a damp chamber. Following three washes in PBS, the slides were coverslipped (mounting solution: 1 ml PBS, 9 ml glycerine, 10 mg p-phenylaminediamine). Slides were viewed on a Zeiss Axioplan microscope.

Antisera.

Anti-ALR antisera #17 and #18 were produced in rabbits that had been immunized with the ALR N-terminal 16 peptide sequence of ALR. Purified immunoglobulin preparations were made from these antisera are referred to here as #17Ig and #18Ig. Two rabbit antisera, #19 and #20 were produced against an internal peptide, MRTQQKRDIKFRED. Other antisera utilized here are: Goat anti-rat transferrin (Nordic Immunological Laboratories, Capistrano Beach, CA, Cat. Code GARa/Tfn, lot 42-285), goat anti-mouse albumin (Nordic Immunological Laboratories, Capistrano Beach, CAD, Cat. Code RAM/Alb, lot 14-787); sheep anti-mouse albumin (Cappel Research Products, Durham, NC, Cat. #0111-0344, lot 26599) rat-anti-mouse F4/80 was kindly provided by Dr. Allan Ezekowitz (Children's Hospital, Boston), goat-anti-mouse fibrinogen (Nordic Immunological Laboratories, Capistrano Beach, CA, Cat. Code GAM/Fbg, lot 3238); swine-anti-rabbit FITC (Nordic Immunological Laboratories, Capistrano Beach, CA; Cat. Code SWAR/FITC, lot #3674); donkey-anti-goat TRITC (Nordic Immunological Laboratories, Capistrano Beach, CA; Cat. Code DoAG/TRITC. lot #3754); goat-anti-rat FITC (Cappel Research Products, Durham, NC; Cat. #1213-1721, lot #1213-1721). Anti-estrogen-receptor antisera were kindly provided by Dr. A. Traish, Boston University Medical Center, anti-carbomoyl phosphate synthetase by Dr. A. Moorman, University of Amsterdam.

Purified recombinant ALR protein.

We utilized recombinant ALR, produced in E. coli, as described by Hagiya et. al. (1994).

Absorptions.

40ul of a mixture of rabbit-anti-rat ALR#17 IgG and goat-anti-mouse albumin (at 1/80 dilution in stock rabbit-anti-rat ALR #17IgG) were incubated for 1 hour at room temperature with 4ul of recombinant ALR peptide solution at 880.00, 88.00, 8.80, 0.88, and 0.088 ug/ml concentrations. These mixtures were incubated at room temperature for 1 hour and left at 4°C overnight. Samples were spun in a micro-centrifuge (Fisher) for 2 minutes, and the supernatants were used for immunofluorescence as described above. Residual anti-ALR #17IgG antibody was assessed on mouse liver by the presence of green cells visualized by FITC anti-rabbit IgG, while anti-albumin antibody was assessed by the presence of red cells visualized by TRITC labeled anti-goat IgG serum. As the anti-ALR and anti-albumin antibodies were mixed together before absorption, each test contained an internal negative control.

Dilution tests.

Two-step two-color immunofluorescence was carried out at 1/1, 1/5, 1/20, 1/80, and 1/360 dilutions of the anti-ALR antisera. Ten liver sections were made, each transferred to a well on a ten-well Teflon-coated microscope slide as described above in the two-step two-color immunofluorescence protocol. Each dilution of the anti-ALR antiserum is applied to two separate liver sections. Four sets of dilution experiments were carried out, with essentially identical results.

Platelet Isolations.

Platelets were isolated from rats. After anesthetizing with ether, the pericardium was opened with a scissors, and blood obtained by direct cardiac puncture with an 18 gauge needle. The freshly obtained blood was immediately anticoagulated with 0.9% sodium citrate, pH 6.0, at a citrate to whole blood ratio of 1:9, and platelet-rich plasma obtained by centrifugation at 1300xg in a swinging bucket rotor (Sorvoll T6000B centrifuge). After transferring the platelet-rich plasma to a fresh tube, a platelet pellet was obtained by centrifugation at 1300xg for 15 minutes. Blood collections and centrifugations were carried out at room temperature. The plasma supernatant was then removed, and the pellet frozen for subsequent sectioning and immunofluorescence, as described above.

# RESULTS

Details of the immunofluorescence visualization of ALR.

Two Anti-ALR antisera, #17 and #18, were produced in rabbits that had been immunized with a synthetic peptide corresponding to the sequence of the 16 N-terminal residues of mouse and rat ALR, and purified immunoglobulin preparations made from these antisera, referred to here as #17Ig and #18Ig. Two rabbit antisera, #19 and #20, were produced against a internal peptide, MRTQQKRDIKFRED. All four antisera are reactive against recombinant ALR in a Western blot, but only #17 and #18 react in an ELISA test (unpublished), suggesting that the antisera #19 and #20 react to an antigenic determinant normally hidden in the intact protein. In agreement with this finding, we found the anti-ALR antisera #17 and #18, as well as #17Ig and #18Ig, to react with structures in the sinusoids and blood vessels of livers of rats and mice, which proved to be platelets (see below), (Figure A-F), while the antisera #19 and #20 revealed no such reactivity in the immunofluorescence assay. Generally, the #17 and #18 anti-ALR whole sera reacted with a somewhat higher background than purified #17Ig and #18Ig reagents. Anti-ALR #17Ig yielded a very slightly greater degree of liver immunofluorescence intensity than Anti-ALR #18Ig, but otherwise the two reagents proved to be remarkably similar.

The ALR seen in the livers of mice and rats could be most brightly visualized when the #17Ig and #18Ig antisera were used at 1/1 dilution, although both were found to still exhibit considerable reactivity at 1/5 dilution. No reactivity for either reagent was was observed at dilutions of 1/20 or less. All of the experiments described in this paper were carried out with #17Ig and #18Ig at 1/1 dilution.

The immunochemical specificity of the ALR immunofluorescence reaction was confirmed by absorption with purified recombinant ALR protein. 40ul mixtures of a rabbit anti-ALR antibody #17Ig and goat-anti-albumin antiserum, were incubated with 4ul of recombinant ALR protein solution at 880, 88, 8.8, 0.88, and 0.088 ug/ml concentrations, and the supernatants then tested for reactivity against ALR and albumin in the liver with a two-step two-color immunofluorescence experiment on mouse liver sections. We found that absorption with 88 and 880 ug/ml purified recombinant ALR completely removed immunofluorescence reactivity against ALR in mouse liver, while absorption with 0.088, 0.88, and 0.88 ug/ml led to progressively dimmer immunofluorescence reactions. As expected, no effect on albumin reactivity (Michaelson, 1993) was found, thus confirming the specificity of the absorbtion test.

In contrast to the distribution of ALR within the sinusoids and blood vessels of the liver, we found that a number of other cellular proteins can be visualized in hepatocytes. First, we found that anti-estrogen receptor antisera react with the nuclei of essentially all hepatocytes in the livers of C57BL/6J strain female mice, a result in agreement with the observations of Yamashita and Korach (1989). Second, the enzyme carbamoylphosphate synthetase has long been known to be located in all hepatocytes except those located in the few rows of cells adjacent to the terminal hepatic venules (Moorman, et al 1988), and we have found exactly this pattern of widespread expression by our methods. Third, we found that the TROMA-I monoclonal antibody, which reacts with a liver-specific mouse cytokeratin, causes immunofluorescence in all mouse hepatocytes. Fourth, as we have described previously, by immunofluorescence, each of a variety of plasma proteins may be visualized in a small number of hepatocytes (Michaelson, 1993). For example, albumin is present in about 1% of the liver's hepatocytes, with each of the other plasma proteins (transferrin, fibrinogen, etc.) present in separate subpopulations of hepatocytes, each of whose abundance reflects the relative rates of synthesis of the corresponding plasma protein (Michaelson, 1993). We see this pattern of expression when two-color immunofluorescence experiments were carried out in which ALR and plasma proteins are visualized simultaneously on the same section. Note in Figures E and F of rat liver, where the ALR positive structures are visualized in green, while the transferrin-containing hepatocytes present in the same field are visualized in red.

A number of negative controls were carried out. Neither normal goat sera nor normal rabbit sera were found to react with any of the tissues examined. None of the antibodies listed in the previous paragraph reacted with the material in the vessels of the liver in a fashion seen with the anti-ALR antisera. In addition, controls utilizing only the FITC/TRITC conjugated secondary antisera without primary antisera, were employed in every experiment described here, and were never found to react.

Immunofluorescence image of ALR in the liver.

We found ALR to be located within the sinusoids and blood vessels of the liver, as seen in Figures A, B and E. In the sinusoids, this material appears as discrete, irregular, threadlike structures, which proved to be platelets (see below). No reaction was seen against the hepatocytes or endothelial cells of the liver.

The immunofluorescence reactivity of the anti-ALR antisera proved to be somewhat brighter when tested against the rat liver than against the mouse liver. In addition, the threadlike patches of ALR positive platelets seen in sinusoids of the rat liver appeared to be somewhat larger, and more abundant, than their mouse counterparts. Since the mouse and rat genes for ALR contain precisely the same amino acid sequence in the peptide against which our antisera were produced (Hagiya et al 1994, Giorda et al 1996), we conclude that difference in reactivity could not be due to differences in antigenicity, but to a species-specific difference in platelet biology.

While the threadlike appearance of the sinusoidal ALR initially led us to suspect that ALR might be present in macrophages (Kupffer cells), this possibility was excluded by comparison of the immunofluorescence image of ALR with that of the macrophage marker, F4/80 (Hume 1983). We found that, in comparison to the ALR positive platelets, which lay within the sinusoids, the F4/80-visualized macrophages were larger and intimately attached to the sinusoids. Furthermore, the F4/80-visualized macrophages bore characteristic nuclei that were not evident in the platelets that are revealed by ALR immunofluorescence.

Isolated blood platelets are ALR positive, and have the same appearance as the ALR seen in the liver and spleen.

Platelets in a pellet made from platelet-rich rat plasma were found to be ALR positive by immunofluorescence (Figures G and H). These blood-derived platelets have the same immunofluorescence appearance as the ALR in the sinusoids of the liver, and the red pulp of the spleen. We also found that ALR appears to be weakly present in megakaryocytes in the bone marrow, the cells that give rise to platelets, although this remains an area for more detailed analysis in the future.

Immunofluorescence image of ALR in the spleen.

In the mouse spleen, ALR was seen as isolated, thread-like structures, which appear to be platelets. Two-color immunofluorescence comparison of ALR with the macrophage marker F4/80 confirmed that macrophages do not carry ALR. The ALR was found to be located principally in the red pulp, where platelets are known to accumulate (Fig. C) (Hume 1983). There were very few such ALR positive structures located in the white pulp of the spleen, and intermediate numbers of ALR patches were seen in the marginal zone surrounding the red pulp (Fig. C).

Immunofluorescence image of ALR in the germ cells of the testes.

We found ALR to be present in the germ cells of the testes, where it had a distinctive granular appearance (Fig. D). The connective tissue that surrounds each seminiferous tubule (the tunica propria) was negative for ALR. Although the frozen sections that we utilized to visualize the ALR did not afford enough histological detail to define precisely the relationship between ALR expression and germ cell development, it generally appeared that the spermatogonia, which reside next to the tunica propria, either contained weak or no expression of ALR, although this could reflect the relatively small amount of cytoplasm in these cells.

Granular ALR was evident in the spermatocytes and spermatids, while a bright filamentous expression was evident on the tails of the spermatozoa that were still within the seminiferous tubule. Whether or not the Sertoli cells contain ALR could not be determined. The occasional mature spermatozoa that we saw that had detached from the seminiferous tubules and resided in the lumen proved not to be positive for ALR, nor were mature spermatozoa from the epididymis.

Immunofluorescence image of ALR expression in the heart, lung, kidney, and skeletal muscle.

Two separate examinations of mouse heart, lung, kidney and skeletal muscle were made by immunofluorescence with anti-ALR antiserum. Although we did not observe the bright immunofluorescence expression seen in the rat liver, spleen, and testes, we did occasionally see ALR in blood vessels in a variety of tissues, apparently in platelets.

ALR during liver regeneration.

We analyzed ALR expression by immunofluorescence in livers from rats at various times after partial hepatectomy (from 2 hours after hepatectomy to six days after hepatectomy) and found no obvious differences in the ALR immunofluorescence images. None-the-less, we suspect that more subtle difference in ALR expression, such as the release of ALR from platelets, may well be occurring during this time.

## DISCUSSION

We have determined, by immunofluorescence, that ALR is present in the platelets in the liver, and indeed, throughout the body. Platelets are known to have active protein synthesis, and contain abundant mRNA, thus accounting for the rough equivalence of ALR protein and mRNA expression (Markovic, 1995, Boyse and Rafelson, 1967). The high level of both ALR protein, as seen by immunofluorescence, and ALR mRNA, as seen on Northern blots (Hagiya, 1994, Giorda, 1996) may be ascribed to the fact that platelets tend to be present in especially high quantity in the sinusoids of the liver (Frank, 1992, Odin, 1988, Kerr, 1995). Platelets are also known to accumulate in the red pulp of the spleen, where we also see ALR by immunofluorescence (Frank, 1992, Odin, 1988). In fact, our immunofluorescence images of ALR in the liver and spleen are remarkably similar to the images found in several previous studies that had examined the distribution of other platelet antigens (Frank, 1992, Odin, 1988).

In the testes, we found ALR to be present in the germ cells, including the spermatozoa, rather than in the somatic tissue. It is of interest that spermatozoa in the testes contain ALR on the tail, but once released into the lumen, spermatozoa become ALR negative. Whether this is due to the partitioning of the ALR to the residual body, which is discarded at about this time Kerr, 1995), or whether ALR becomes masked or degraded, remains to be determined.

No other vertebrate proteins are known which are homologous to ALR, although it does bear homology to a yeast protein, ERV1, which plays an essential role in the control mitochondrial gene expression (Hagiya, 1994, Giorda, 1996). The ERV1 protein was identified by Lisowsky as the gene product of the *pet-ts492* mutation in *Saccharomyces cerevisiae* (Lisowsky, 1992, 1994, Lisowsky, et al 1995). Lisowsky found *pet-ts492* to be a nuclear mutation, which causes the loss of mitochondrial gene transcripts, and eventually, the loss of the mitochondrial genome (Lisowsky, 1992, 1994, Lisowsky, et al 1995). Since the defect in mitochondrial gene expression caused by mutation of ERV1 in yeast can be complemented by transfection with the human ALR gene, we know that the human and yeast genes are not just homologous in sequence, but homologous in function as well (Lisowsky, et al 1995). In this regard, it is of interest that our immunofluorescence images, which have revealed ALR to be located in granular structures in spermatogonia, spermatocytes, and spermatids, and in the sperm tails, where the mitochondria are located, are consistent with, but not conclusive of, a mitochondrial association in mammalian cells.

The immunofluorescence findings raise the question of why ALR, a protein that appears likely to function in the control of mitochondrial gene expression, would be present only in platelets and spermatozoa. Both cells types have vigorous mitochondrial gene expression. In fact, platelets and spermatozoa are the only two types of mammalian cells that require vigorous expression of the mitochondrial genome in the absence of functioning nuclear gene expression. Perhaps ALR in the testes and platelets provides a gene control function normally shared by the genomes of the nucleus and the mitochondrion. Both spermatozoa and platelets are also entities with unusually high requirements for mitochondrial activity. As Holmsen has written "stimulation and execution of platelet responses requires the turnover of cytoplasmic ATP that is higher than in most other cells; when it is lower, platelet responses are powerfully inhibited" (Holmsen 1985).

The immunofluorescence finding that ALR is expressed in spermatozoa is of special interest in light of the fact that the mouse ALR gene maps to a genetic region long known for its effect on sperm function: the *T/t*-region of chromosome 17 (Giorda, 1996). This region is marked by two types of genes: conventional dominant T mutant alleles, which are point mutations, and which are lethal when homozygous, and cause a short tail when heterozygous, but have no effect on sperm function, and recessive t-mutants, which are actually genetic changes that stretch over a wide range of chromosome (Bennett, 1975, Silver 1985, 1993, Klein and Hammerberg, 1977). t-mutants are either lethal, or male sterile, when homozygous (*t/t*) or doubly heterozygous ($t^a/t^b$). In one of the most unusual features of this system, *t/+* male mice are often found to transmit their mutant *t* alleles to many more than the expected 50% of their offspring. This violation of Mendelian expectation, called transmission ratio distortion, is apparently caused by the "poisoning" of + germ cells by *t*-germ cells. The same interaction is presumed to lead to sterility, by mutual germ cells destruction, in viable, but sterile, homozygotes ($t^v/t^v$) and double heterozygotes ($t^a/t^b$) (Herman et al 1990).

Several reports have suggested that *t*-mutant mice may have defects in mitochondrial function. Thus, Hillman and colleagues have found that $t^6/t^6$ homozygous embryos contain cells with defective mitochondria (Hillman, 1975), as seen by electron microscopy, while Blake has found that liver from $t^6/+$ heterozygous mice have 40% of activity of the mitochondrial enzyme cytochrome c oxidase as in wild-type liver (Blake 1977) Indeed, *t*-mutations appear to have much the same effect on mitochondrial function in mice that ERV1 mutations have in yeast (Lisowsky, 1992, 1994, Lisowsky, et al 1995).

The transmission ratio distortion of *t*-alleles has been found to be determined by a number of genes scattered across the expanse of the *T/t* region, and are clearly separable from the genes that lead to embryonic death and the short tail phenotypes (Silver 1985, 1993, Lyon, 1990). In recent years, there has been an active search for genes in the *T/t* region genes that might explain the effect of *t*-mutations on sperm function. These investigations have resulted in the identification of a variety of chromosome 17 genes that are expressed predominantly in the testes, although none have been unambiguously shown, at least yet, to be causally related to *T/t* region transmission ratio distortion phenotype (Cebra et al, 1991, Ha et al 1991, Lader et al 1989, Mazarakis et al 1991, Rappold, et al 1987, Schimenti et al 1988, Silver 1993, 1985, Sorrentino et al 1988, Yoem et al 1992 Silver et al 1983, Huw et al 1995, O'Neil and Artzt 1995, Willison et al 1988). Taken together, the ALR chomosomal location in mice, the phenotype of ALR/ERV1 mutations in yeast, and our immunofluorescence findings, raise the question of whether a mutated form of ALR, causing a defect in spermatozoan mitochondrial activity could contribute to the transmission ratio distortion effect seen in *t*-mutant mice. Such a possibility would require that in *t/+* mice, ALR enters all germ cells, but inactivates mitochondrial gene expression only in "+" bearing germ cells. Such a model would have to assume a degree of insensitivity to this poisoning on the part of the "*t*" bearing germ cells, due to a second "antidote" gene, but this is entirely consistent with the genetic data, which tells us that multiple genes in the *T/t*-region contribute to the transmission ratio effect (Silver, 1985, 1993, Lyon, 1990). Of course, such a possibility remains to be tested experimentally.

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## FIGURE LEGENDS

A) ALR (green) in the sinusoids of rat liver (50X).

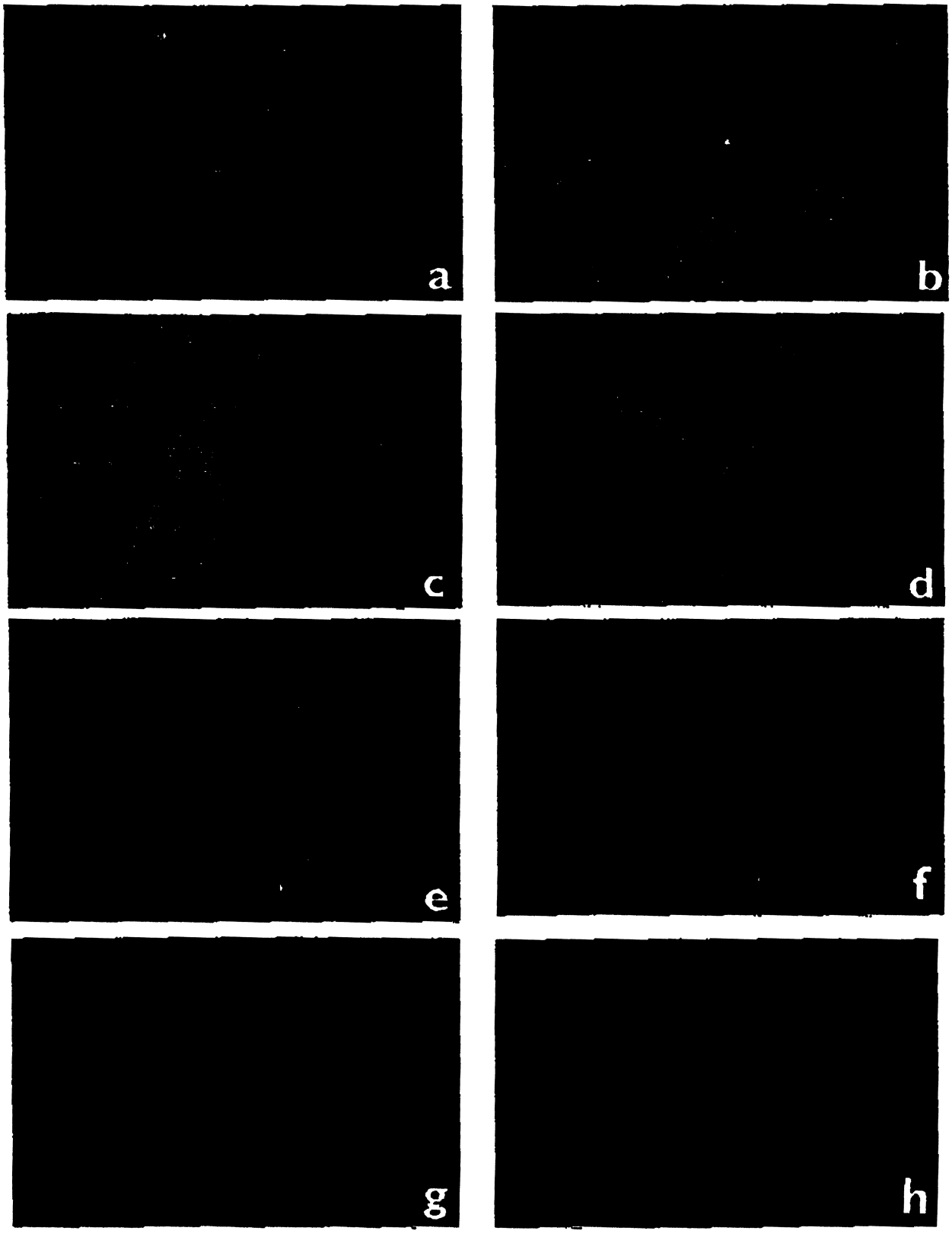
B) ALR (green) in the blood vessels and sinusoids of rat liver ( 100X).

C) ALR (green) in mouse spleen (110X).

D) ALR (green) in mouse testis (100X).

E, F) ALR (green), Transferrin (red) (50X). Note that ALR is located in the sinusoids, but not in the hepatocytes, while transferrin is located in just a single hepatocyte in this field, in agreement with previous findings (5), which have shown that a large number of plasma proteins are each located in a small, separate, subpopulation of the liver's hepatocytes (16,17).

G, H) ALR immunofluorescence carried out on platelets in a pellet created from platelet rich rat blood.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