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Immunofluorescence of Mineralocorticoid Receptors in Peripheral Lymphocytes: Presence of Receptor-like Activity in Patients with the Autosomal Dominant Form of Pseudohypoaldosteronism, and its Absence in the Recessive Form

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Pseudohypoaldosteronism is a syndrome characterized by salt wasting and a failure to thrive due to the resistance towards the action of aldosterone. Aldosterone levels and plasma renin activity are extremely elevated and aldosterone binding sites in peripheral mononuclear leukocytes have regularly shown to be reduced or absent. Sporadic as well as familial cases have been identified and an autosomal dominant as well as an autosomal recessive mode of inheritance has been described. A defect in the aldosterone receptor has been postulated, however, molecular genetic analysis in selected patients has not revealed a mutation in the sequence of the coding region of the cDNA of the mineralocorticoid receptor gene. In the present study we have used a fluorescence-labeled antibody to detect possible receptor expression in monocytes from patients with various clinical forms of pseudohypoaldosteronism. Patients with the sporadic as well as with the autosomal dominant form were clearly immunopositive despite being negative in terms of aldosterone receptor binding. In contrast in two patients with the autosomal recessive form there was no detectable receptor protein, consistent with the results obtained in the aldosterone binding studies. These results suggest that the pathogenesis of pseudohypoaldosteronism is heterogeneous not only regarding the mode of inheritance but also in terms of receptor binding. Thus, in a subgroup of patients the inability of the receptor to bind ligand may be due to a defect involving other, probably cellular factors rather than a deficiency or a defect in the mineralocorticoid receptor system itself.

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INTRODUCTION

The syndrome of pseudohypoaldosteronism is caused by a resistance towards the action of mineralocorticoids resulting in salt wasting and a failure to thrive. Aldosterone levels and plasma renin activity are extremely elevated and mineralocorticoid receptors in peripheral mononuclear leukocytes are consistently absent or reduced in sporadic and familial forms of pseudohypoaldosteronism [1]. In the familial cases autosomal dominant and autosomal recessive forms of inheritance can be differentiated, on both biochemical and clinical grounds [2]. The autosomal dominant form is characterized by asymptomatic carriers, and by relatively mild clinical symptoms in affected individuals compared to the patients with the autosomal recessive form of inheritance [2, 3].

Recent studies have shown that the cDNA sequence of the mineralocorticoid receptor gene is normal in both the index case of the disease and in another, unrelated autosomal dominant patient [4, 5]. These results cause some doubt on the original hypothesis that a defect in the mineralocorticoid receptor is indeed

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responsible for the mineralocorticoid resistance in these patients.

In order to further evaluate the presence of mineralocorticoid receptors in patients with various clinical forms of pseudohypoaldosteronism immunofluorescence localization of receptor protein was performed using a polyclonal antibody raised against a fusion protein containing a 167 amino acid fragment of the human mineralocorticoid receptor [6].

MATERIALS AND METHODS

Patients

Patient 1, the first patient reported with pseudohypoaldosteronism, was originally described by Cheek and Perry [7], and was subsequently shown to lack mineralocorticoid receptors in monocytes by the ligand binding assay [1]. Both plasma aldosterone and renin remained substantially elevated in this patient, despite his clinical improvement [4].

Patient 2 is a 2 month old female infant who presented with failure to thrive and electrolyte abnormalities characteristic of aldosterone deficiency (hyponatremia, hyperkalemia and metabolic acidosis). Congenital adrenal hyperplasia was excluded and the diagnosis of pseudohypoaldosteronism was made on the basis of elevated aldosterone levels, high plasma renin activity and the absence of aldosterone binding sites in mononuclear leukocytes. Over 3 days of treatment with fludrocortisone sodium excretion remained unchanged, substantiating aldosterone resistance. Both parents, as well as the patient's 3 year old sister, had normal biochemical findings and aldosterone binding in monocytes.

Patient 3 is the second affected child in a family with the autosomal dominant form of pseudohypoaldosteronism. His mother has been shown to be an asymptomatic carrier. The presence of pseudohypoaldosteronism in this child was verified by biochemical measurements, and no aldosterone binding was found in the monocytes.

Patients 4 and 5 are children of a consanguineous marriage with the autosomal recessive form of pseudohypoaldosteronism. These two siblings have been shown previously to have greatly reduced aldosterone binding in their monocytes [1]. The biochemical and clinical findings in both siblings and their parents are characteristic of the autosomal recessive form of pseudohypoaldosteronism. Both patients still require high doses of oral sodium supplementation daily, and both are frequently hospitalized during minor intercurrent infections [8]. In contrast, both parents are normal in terms of their biochemical and hormonal parameters [2].

Healthy controls were recruited from laboratory staff who had previously volunteered for determination of their mineralocorticoid receptor levels, which had been shown to be normal [1].

Immunofluorescent localization of leukocyte mineralocorticoid receptors

Mononuclear leucocytes from about 10 ml of heparinized blood were separated by Ficoll gradient centrifugation. Isolated cells were washed twice, resuspended, spread on a gelatinized slide and left to dry for 10 min at room temperature. The cells were then fixed for 10 min in pure methanol (analytic grade), and again left to dry at room temperature for 20 min. Slides were pretreated with commercial goat serum diluted with Tris buffer 1:10, and then incubated at room temperature with the specific anti-receptor antibody at a dilution of 1:200 for 45 min. To evaluate nonspecific binding a set of slides, pretreated identically, were incubated for 40 min with commercial nonimmune rabbit serum. The slides were carefully rinsed with Tris buffer, and incubated for 45 min with a commercial biotinylated goat anti-rabbit antibody at a dilution of 1:200, and then rinsed with Tris buffer. Fluorescence was visualized with a streptavidin fluorescent antibody (dilution 1:200) incubated for 45 min in the dark before microscopy. The whole procedure was performed at room temperature.

RESULTS

When mononuclear leukocytes from control subjects were incubated with the MR4 antibody and the cells examined immunoreactivity appeared as a bright stain in the cell cytoplasm; the nuclei, which occupy the majority of the cell, remained unstained. Parallel exposure of control monocytes to normal rabbit serum showed background staining only (Fig. 1).

Specific immunofluorescence was also observed when the monocytes of Patient 1, the index case of pseudohypoaldosteronism, were analyzed (Fig. 2). This subject is a sporadic case, and has previously been shown to have no aldosterone binding sites [1]. Patients 2 (sporadic) and 3 (from an autosomal dominant family) both showed positive mineralocorticoid receptors by immunofluorescence (Fig. 3), while aldosterone binding by the radioreceptor assay was negative.

Not all patients with pseudohypoaldosteronism, however, showed immunoreactive mineralocorticoid receptors. Patients 4 and 5 did not demonstrate specific immunofluorescence (Fig. 4). The mother of these two patients [1] clearly showed immunofluorescent staining of her monocytes (Fig. 5).

DISCUSSION

The detection of mineralocorticoid receptor-like immunoreactivity in normal monocytes is consistent with the demonstration of high affinity aldosterone binding sites in these cells [1]. Immunoreactivity was cytoplasmic, and was observed as a corona around the periphery of the cell, reflecting the small cytoplasmic space surrounding the large nucleus. The absence of

nuclear immunofluorescence does not appear to reflect an inability of the antibody to recognise a nuclear mineralocorticoid receptor, since the MR4 antibody has previously been used to demonstrate both cytoplasmic and nuclear localization of the receptor [6].

In the present study the exclusive localization of mineralocorticoid receptor in the cytoplasm may reflect dissociation of the ligand from the receptor, and the subsequent recycling of the receptor from the nucleus; purification of mononuclear cells on Ficoll gradients and the subsequent washing steps could cause such a loss of ligand from the cell.

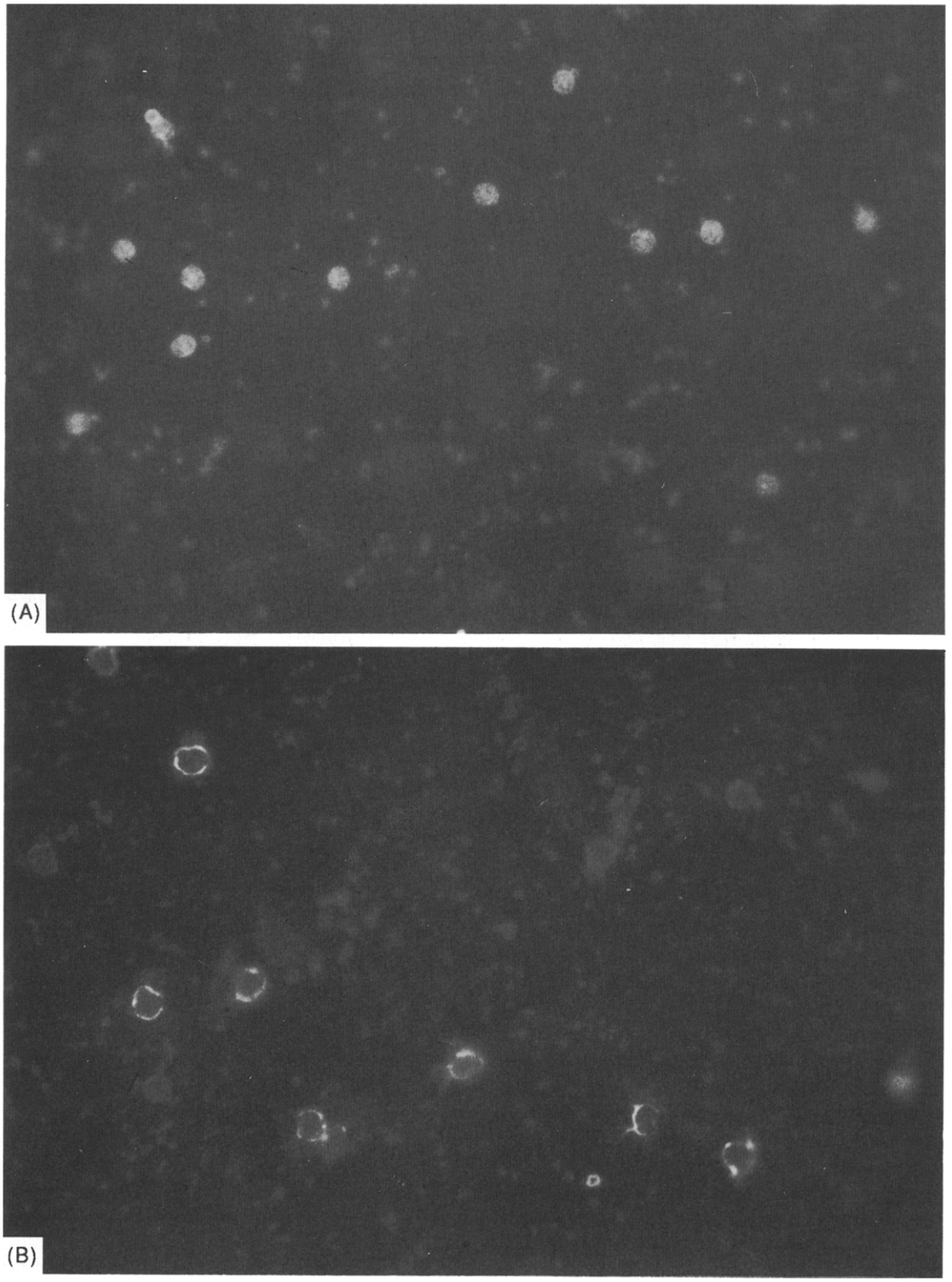

Fig. 1. (A) Labeling of control monocytes using normal rabbit serum which demonstrates background staining only. (B) Positive immunofluorescent labeling of control monocytes using the MR4 antibody. The immunoreactivity appears as a bright stain in the cell cytoplasm.

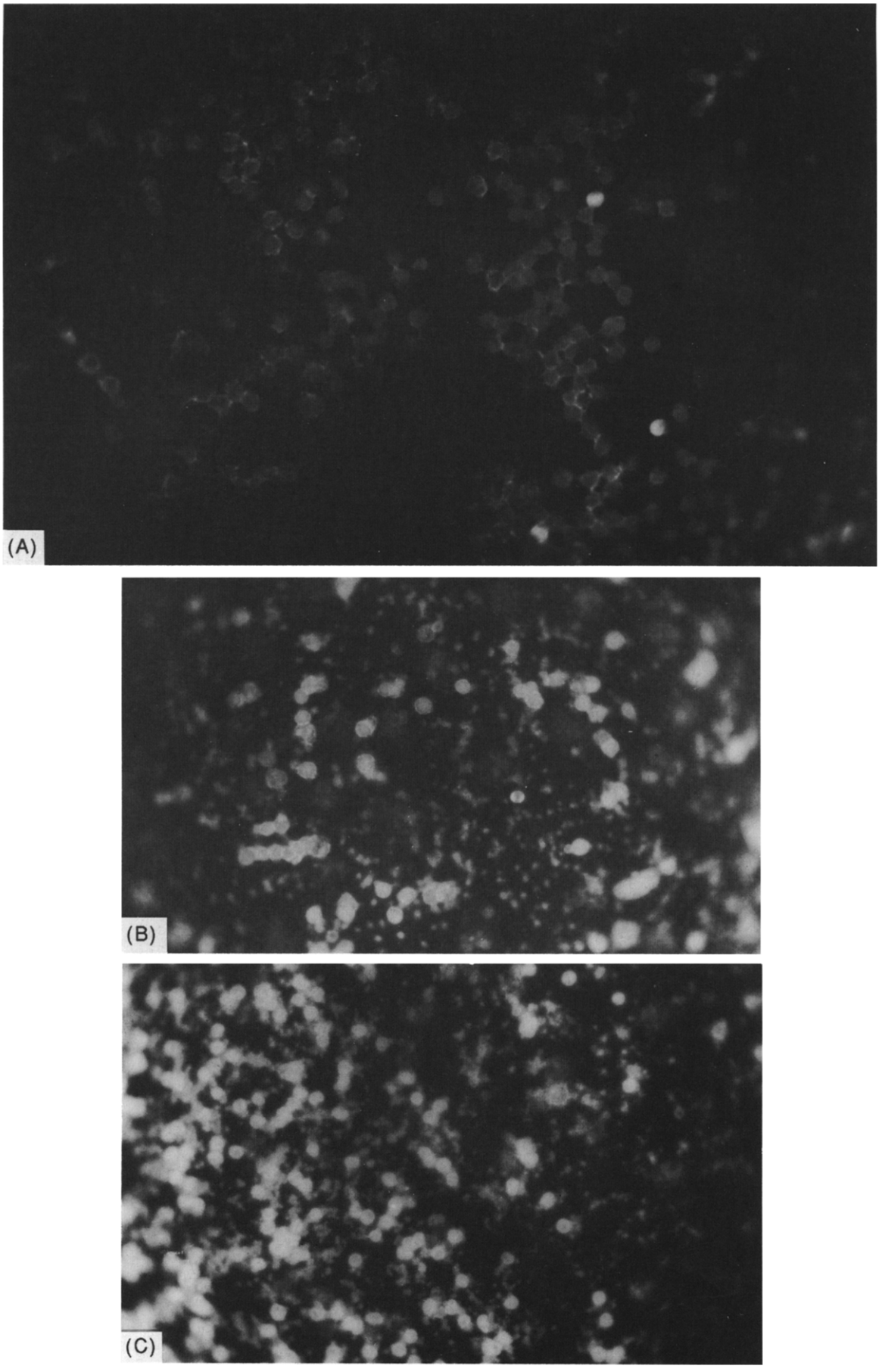

Fig. 2. Positive immunofluorescent labeling of monocytes. (A) From Patient 1, (B) from Patient 2, (sporadic cases) and (C) from Patient 3 (autosomal dominant case) using the MR4 antibody. The immunoreactivity is clearly positive and appears as a bright stain in the cell cytoplasm.

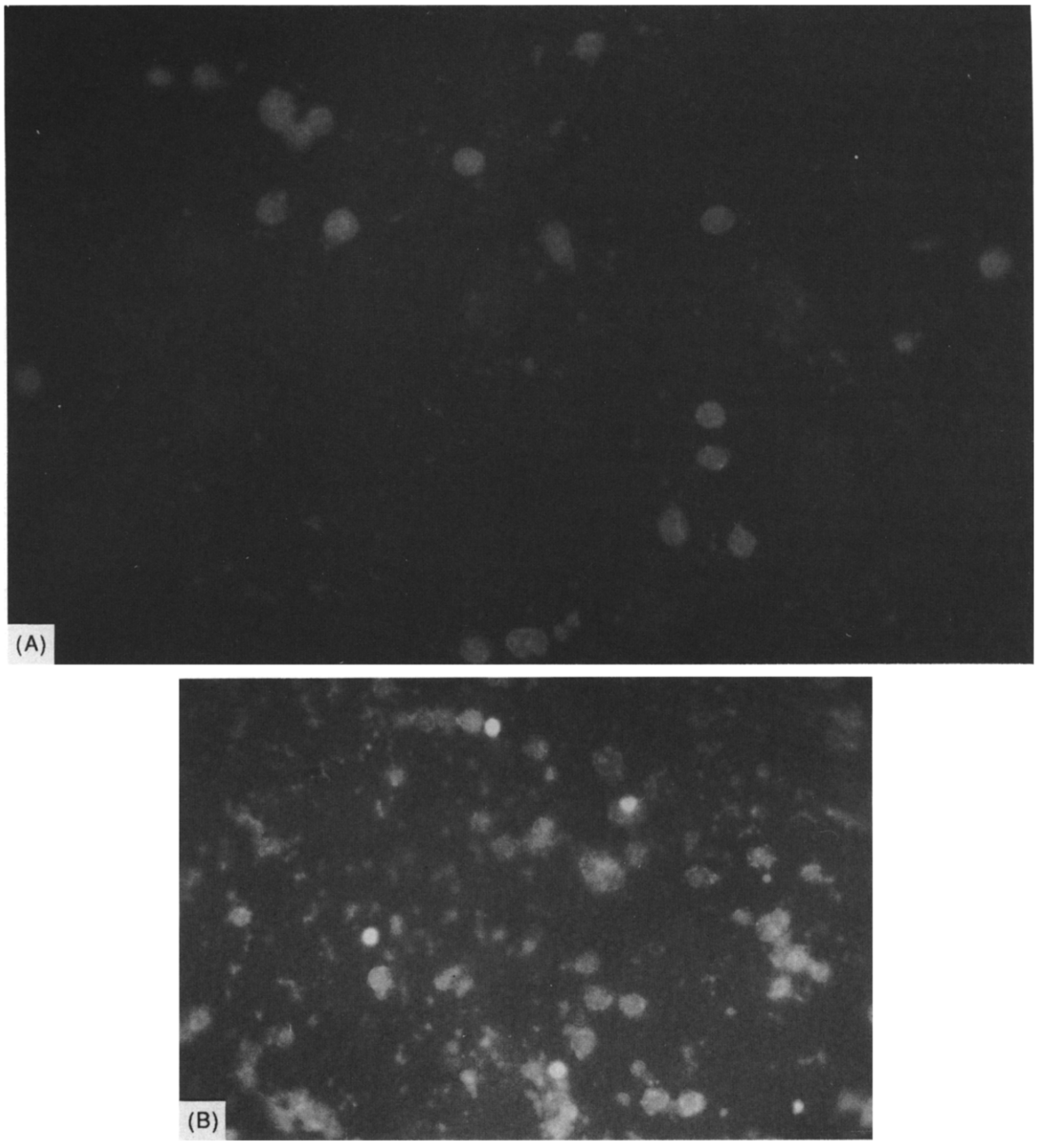

Fig. 3. Negative immunofluorescent labeling of monocytes. (A) from Patient 4 and (B) from Patient 5 using the MR4 antibody. Background staining only can be seen, see Fig. 1(A).

The significant finding of the present study is the observation that there is a subgroup of patients with pseudohypoaldosteronism who demonstrate mineralocorticoid receptor-like immunoreactivity in their mononuclear cells despite being negative in terms of aldosterone receptor binding [1]. In the patients studied here, two with the sporadic form of the disease (Patients 1 and 2) and one patient with the familial dominant form (Patient 3), immunofluorescent localization of the receptor was clearly positive, and indistinguishable from normal controls.

In contrast, in the two patients with the autosomal recessive form of the disease (Patients 4 and 5) immunofluorescent localization of mineralocorticoid receptor was unequivocally negative. While Patient 4 has consistently proven to be negative by the radioreceptor binding assay, Patient 5 displayed very low but measurable mineralocorticoid receptor-binding, which was more than 2 SD below control levels [1]. Thus the presence or absence or immunofluorescence does not correlate with the aldosterone receptor binding data. Whether there is a correlation with the results of the molecular genetic analysis remains to be seen. To date no analysis has been performed in patients with the autosomal recessive form.

It is of interest to note that there is no correlation between the number of aldosterone receptor binding sites and the severity of salt loss [2, 8, 9]. Low or absent

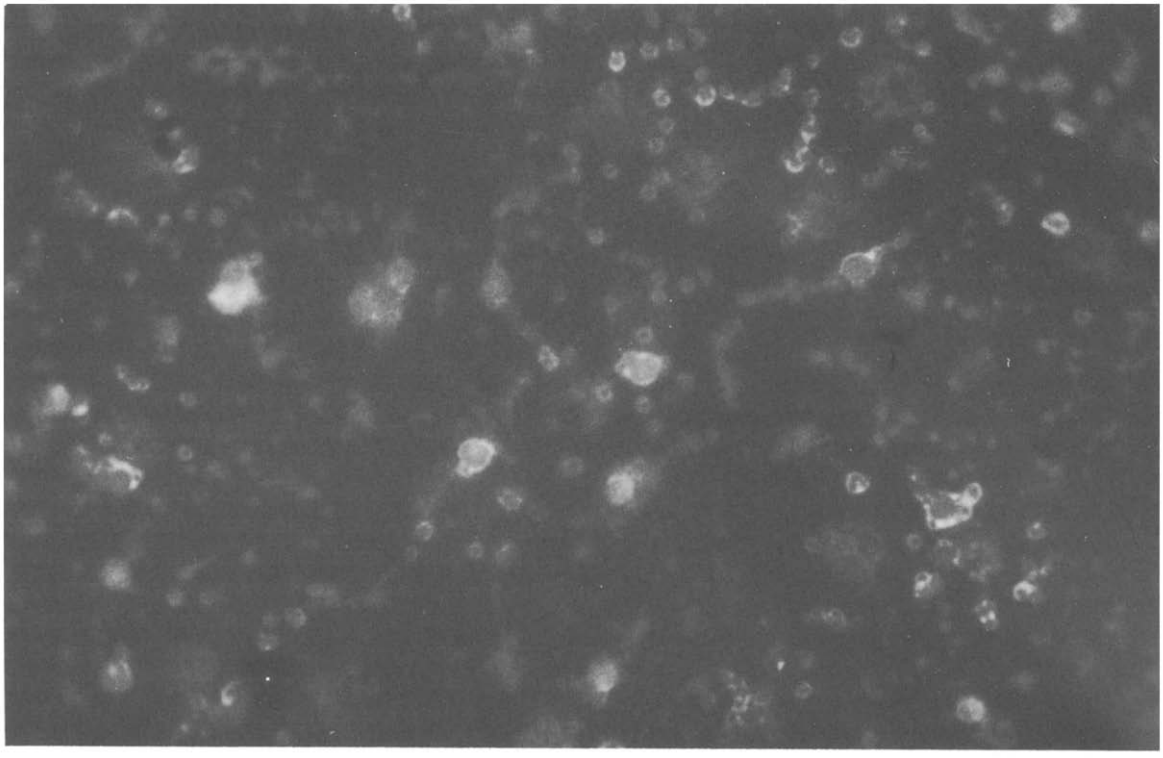

Fig. 4. Positive immunofluorescent labeling of monocytes from the mother of Patients 4 and 5 using the MR4 antibody. The immunoreactivity is clearly positive and appears as a bright stain in the cell cytoplasm.

aldosterone binding sites in mononuclear leukocytes have been regularly shown to be a characteristic finding in patients with pseudohypoaldosteronism. However patients with the autosomal dominant and sporadic forms display only mild salt loss and may eventually overcome the need for sodium supplementation, whereas patients with the autosomal recessive form of the disease have similar aldosterone receptor binding results but require continual sodium supplementation and are frequently hospitalized due to salt-losing crises accompanying minor infections [8, 10].

The pathogenesis underlying pseudohypoaldosteronism thus appears to be multifaceted and complex. Mineralocorticoid receptors, as determined by radioreceptor binding and immunofluorescence techniques, seem to be absent in a subgroup of patients. However there are patients in whom receptor binding is absent but immunofluorescence is clearly positive. This finding correlates with the absence of a mutation in the cDNA sequence of the mineralocorticoid receptor gene which has been shown at least for one of the patients studied (Patient 1).

It has to be concluded that mineralocorticoid receptors are present at least in some patients with pseudohypoaldosteronism. We might speculate that the compromised binding may reflect a defect in one or more associated proteins necessary for steroid action. Steroid receptors exist as oligomeric protein complexes in association with as many as seven other proteins (for a review see Ref. [11]). Significantly, heat-shock proteins are required for strong ligand binding to the glucocorticoid receptor and the same may hold true for the closely related mineralocorticoid receptor [12]. Associated proteins may thus act as cytoplasmic chaperones to stabilize the receptor until ligand is bound. It may thus prove fruitful to examine receptor-associated proteins in patients with pseudohypoaldosteronism to further elucidate not only the pathogenesis of this disorder but to better understand the mechanisms of mineralocorticoid action.

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