CYTOSKELETON PROTEINS IN CHEDIAK-HIGASHI SYNDROME

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The Chediak-Higashi syndrome (CHS) is a rare genetic disease characterized by the presence of abnormally large cytoplasmic masses in all body granule producing cells. The precise molecular mechanism for this disease is still unknown. We report studies on cytoskeleton protein components in peripheral blood leukocytes and in relation to capping of concanavalin A receptors. Marked increase in spontaneous concanavalin A (Con A) capping and a lower endocytic rate were observed; however, cytochalasin B did not modify this spontaneous capping. Microfilament or associated proteins in CHS cells did not show differences with those of control subjects. Cytoplasmic distribution of tubulin and microtubule associated proteins were similar to that of controls. However, we noted an atypical cellular distribution to tyrosinolated tubulin and to anti MAP- 1 antibodies; and by SDS-gel electrophoresis a diminished intensity of tyrosinolated tubulin was also detected. In CHS leukocytes a decrease reactivity to antibodies to band 3 transmembrane protein was observed.

Key words: Chediak-Higashi syndrome. Cytoskeleton. protein band 3

ESTUDIOS DEL CITOESQUELETO EN EL SINDROME DE CHEDIAK-HIGASHI

El Síndrome de Chediak-Higashi (CHS) es una rara enfermedad genética caracterizada por la presencia de grandes masas citoplásmicas en todas las células del cuerpo productoras de gránulos. El mecanismo molecular de esta enfermedad es todavía desconocido. En este artículo se describen estudios sobre los componentes del citoesqueleto en leucocitos de sangre periférica y en relación con el fenómeno de coronamiento (capping) de receptores para concanavalina A. Se observó un acentuado coronamiento espontáneo de estos receptores y una disminución de la velocidad de endocitosis, pero la citocalasina B no modificó este coronamiento espontáneo. Los microfilamentos o proteínas asociadas, en las células de este síndrome no mostraron diferencias con aquellas de los sujetos controles. La distribución citoplásmica de la tubulina y de proteínas asociadas a microbulos fue similar a aquella de los controles. Sin embargo, se apreció una distribución atípica de la reacción de los anticuerpos contra la tubulina tirosinada y para la proteína MAP- 1. Por electroforesis en gel-SDS se apreció una disminución en la intensidad de la tubulina tirosinada. Una reducción de la reactividad de los anticuerpos contra la proteína banda 3 se pudo apreciar en las membranas de eritrocitos de pacientes con este síndrome.

Palabras clave: Citoesqueleto. Proteína banda 3. Síndrome de Chediak-Higashi.

Abbreviations: COMT: Center of microtubule assay. CHS: Chediak-Higashi syndrome. PMN: Polymorphonuclear neutrophils.

INTRODUCTION

The Chediak-Higashi syndrome (CHS) [1] is a rare genetic, autosomal recessive, disease defined by the presence of large cytoplasmic granules in all body granule producing cells, and characterized by increased size of lysosomes, secretory and pigment granules. The main clinical, and features are due to functional alterations of neutrophils, melanocytes and platelets [2-4]; also, an altered cytotoxic T lymphocytes (CTL) and NK cell lytic activity has been well documented.

The precise or basic cell and molecular defects caused by the Chediak-Higashi gene are still unknown and several mechanisms have been postulated [5-9] being

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one of the proposed hypothesis an impaired microtubule function causing a disorder assembly and defective interactions with membranes.

The main reported evidence for a defective microtubule assembly and/or function in these patients has been the spontaneous capping of cell surface concanavalin A receptors by CHS leukocytes and its correction by dibutyryl cGMP and cholinergic agonists [5-10]. However, immunofluorescence and electron microscopy analysis, did not reveal any abnormal qualitative microtubule and microfilament cytoplasmic distribution [11-13]. Elevated levels of tyrosine incorporation in CHS neutrophils and higher amounts of tyrosine fixed to tubulin have been described [14]. These levels could be increased by the stimulatory effect of the chemoattractant fMet-Leu-Phe. Ascorbate treatment of CHS PMN was able to reduce the rate and extent of post-translational tubuline tyrosine in CHS PMN induced by the fMet-Leu-Phe peptide both *in vivo* and *in vitro*. These results suggested that changes in redox state in stimulated PMN may be linked to the induction of tubulin tyrosination.

Since it has become clear that association between cytoskeleton and membranes plays a significant function in signal transduction pathways [15-17], we have studied membrane components (band 3 protein) [18,19] and the cytoskeleton, and associated proteins in leukocytes from Chediak-Higashi patients and their relation to membrane receptor mobility.

MATERIALS AND METHODS

Studies were performed in 7 CHS patients, 5 females and 2 males, with ages between 6 months and 19 years. All of them were born in Pregonero or nearby small communities in the State of Táchira, Venezuela, a cluster of "endemic", Chediak-Higashi syndrome [4]. Parents, brothers and sisters of the patients and healthy subjects, as controls, of the same area were also studied. Chediak-Higashi syndrome diagnosis was established by clinical findings and by the presence of large intracytoplasmic azurophilic granules in polymorphonuclear neutrophils in peripheral blood smears.

Isolated peripheral blood lymphocytes and neutrophils were obtained by Ficoll-Hypaque gradient centrifugation. Purified neutrophils were obtained by mild hypotonic shock treatment of isolated cells.

The following specific monoclonal and polyclonal antibodies to cytoskeleton membrane proteins were tested: phalloidin-rhodamine, antibody to actin, anti-polymeric actin, anti-vinculin, anti-actinin, anti α-tubulin, anti-8-globulin (Amershan Lab, USA), and to acetylated and tyrosilated tubulin (SIGMA, USA). Monoclonal JA2, and anti MAP-1 antibody was obtained from Dr. Jan de Mey (Institute J. Monod, Paris France). The polyclonal antibody directed to the last 15 aminoacids of the transmembrane fragment of the erythrocyte band 3 membrane protein, was a gift from Dra. Ana M. García, (EISA Laboratories, Boston, USA). Rhodamine-labelled monoclonal anti-mouse immunoglobulins were commercially obtained (Coulter, USA). Antibodies to globular actin, actinin, vinculin, MAP-1 and to the band 3 transmembrane fragment were diluted 1:500; tyrosinated tubulin 1:800; phalloidin rhodamine 1:50; and anti-acetylated tubulin 1:10.

Distribution pattern of cytoskeleton membrane proteins in CHS cells was studied by indirect immunofluorescence assay. To such end Ficoll-Hypaque isolated cells were layered on polylysine coated cover slips and fixed with 3% paraformaldehyde (PAF) for 10 min. Cells were then washed with PBS-NH4Cl 50mM (SIGMA) and permeabilized with 0.1 Triton X-l00 (Sigma, USA) for 2 min after which, cells were washed with PBS-gelatin. Cells were then incubated with antibody dilution for 30 min, washed with PBS-gelatin three times and incubated with rhodamine labelled anti mouse monoclonal antibodies for 30 min. After washing with PBS-gelatin and then with PBS three times more, cells were mounted in Moviol (Hoechst, Germany). Large intracytoplasmic granules were visualized by staining them with 0.01 % acridine orange (Merck, Germany). Antibody reactions were visualized in a Leitz epifluorescence microscope.

Concanavalin A receptor mobility was studied at 2, 5, 10, 15 and 30 min. Cells ($2x10^6$) were incubated with Con A-FITC, 10 mg/ml in PBS at 4°C; the cells were brought and kept for the desired time at 37°C; the reaction was stopped by the addition of 3% PAF, for 10 min and after washing three times mounted in Moviol and examined by fluorescence microscopy. The presence and/or distribution of cytoskeleton membrane proteins in relation to cap formation, were detected after 15 min Con A-FITC incubation at 37°C.

The effect of Colchicine, Cytochalasine B and sodium azide on the membrane receptor mobility in CHS leukocytes, was examined by incubating $2x10^6$ cells with 10^{-6} M Colchicine, 10 µg of Cytochalasine B or 20 mM sodium azide for 30 min at 37°C; after washing with PBS, cells were incubated with Con A-FITC for 2 min at 4° C, washed three times, fixed with 3% PAF, washed with PBS, mounted in glycerol and observed under fluorescence microscope.

The analysis of cytoskeleton cell proteins electrophoretic pattern in CHS neutrophils and controls was determined by SDS polyacrylamide gel electrophoresis. Ficoll-Hypaque isolated neutrophils, 10^7 cells/ml, were incubated at 4°C for 30 min with lysis buffer pH 8.3, composed of 1 % Nonidet P-40, 0,5% sodium deoxycho-

late in Tris-EDTA buffer. After centrifugation at 7000g for 20 min to eliminate the nucleus, the supernatant was treated with 1 mM PMSF, phenylmethyl sulphonyl fluoride, a protease inhibitor, in ethanol. Electrophoresis was performed in 10 % SDS-PAGE in Mercaptoethanol containing Tris Buffer and in a Mini-Protean 11 cell (BioRad, USA) at 100 volt in Tris-base [20]. Identification of tirosinated tubulin was performed after protein transfer to nitrocellulose acetate membranes (Bio-Rad, USA), and by their reaction with monoclonal antibody to tyrosinated tubulin. To such end, membranes were first incubated for 24 hrs in a blocking solution composed of 3 % BSA Tris-HCL buffer solution; and after their washing, were incubated with anti tirosinated tubulin monoclonal antibody at 1:300 dilution in the blocking buffer solution. After washing 3 times, membranes were incubated for 1 hr in a Protein A-peroxidase solution (Sigma, USA) at 1:100 dilution. Reaction was developed with Diaminobencidine (DAB, Sigma) in H_2O_2.

RESULTS

Direct and indirect immunofluorescence studies exhibited differences in the distribution pattern of some cytoskeleton and transmembrane proteins of CHS cells as compared to controls (*Table 1*).

Cell distribution of the anti alfa-tubulin or anti beta-tubulin antibody reactivity in both, normal and CHS mononuclear cells and neutrophils, was variable with no special association and the center of microtubule organization was observed in some samples.

Tyrosinated tubulin antibody reaction in neutrophils from CHS patients did not show specific cytoplasmic location; visualizing a diffuse pattern of immunofluorescence; this in contrast with that of controls and CHS heterozygous cells where this reaction was found to be associated to cytoplasmic granules (*Figure 1*). Interestingly enough, the 19 years old CHS patient showed a cell pattern similar to that of controls. Anti acetylated tubulin antibody gaved a diffuse reaction pattern and no differences among the three studied groups were evidenced.

In CHS Con A cap forming lymphocytes and neutrophils, tyrosinated tubulin reaction also differed from that of control cells: CHS leukocytes showed a cytoplasmic diffuse pattern of , but, in controls was observed in the cap region. In cells from the 19 years antibody reactivity old CHS patient, again, a normal cell type pattern was observed. Microtubule associated protein MAP-1 , detected by the monoclonal antibody JA2, was frequently observed to be associated with cac regions in

Table 1. Cell distribution patterns of cytoskeleton and transmembrane proteins in Chediak-Higashi syndrome and control lymphoid cells

Antibodies to Proteins	Controls	Distribution patterns in:	
		CHS Patients	CHS Heterozygous
Tyrosinated tubulin	Associated to granules	Not associated	Associated to granules
Acetylated tubulin	Diffuse	Diffuse	Diffuse
Ttubulin -α	Variable distribution, with no special association COMT was observed in some special samples.		
Tubulin-β	Variable	Variable	Variable
MAP-l	Variable	Cap region	Variable
Globular actin	Membrane associated	Membrane associated	Membrane associated
Phalloidin	Membrane associated	Membrane associated	Membrane associated
Actinin	Membrane associated	Membrane associated	Membrane associated
Vinculin	Membrane associated	Membrane associated	Membrane associated
Band 3	Strong, diffuse fluorescence	Faint fluorescence	Faint fluorescence

CHS cells, whereas, it showed a variable distribution in cells from normal subjects and heterozygous CHS subjects.

Microfilament protein components did not show differences in either, distribution or fluorescence intensity, between CHS patients and control subjects. In both, globular and polymeric (phalloidin) actin, a peripheral distribution closely linked to the cell membrane was observed; and in some CHS leukocytes association with membrane patches and caps was noted. Similarly, actinin and vinculin were found to be membrane associated. Also, no significant differences were detected in the beta-tubulin cell distribution. Anti alpha-tubulin reactivity was also similar in CHS and controls showing no special association; a diffuse distribution was observed in mononuclear cells and neutrophils of both controls and CHS patients; with, in some cells, association to the center of microtubule organization.

Anti Band 3 antibody showed a faint immunofluorescence reaction in CHS cells as compared to that of controls (*Figure 2*). In the 19 years old CHS patient, the fluorescence intensity was similar to that of controls.

The Con A cell membrane receptor interaction in lymphocytes and neutrophils was markedly different between CHS patients and controls. At 4°C a uniform cell membrane pattern was observed in controls and CHS heterozygous; however, capping was already formed in 90% of the cells, neutrophils and lymphocytes, from CHS patients (*Figure 3*). At 37°C, fluorescence in normal cells changed from patches to capping and finally to endocytosis; this, endocytosis by CHS cells was delayed, being still observed after 1 hour incubation at 37°C (*Table 2*). It should be noted that the 19 years old CHS patien behaved

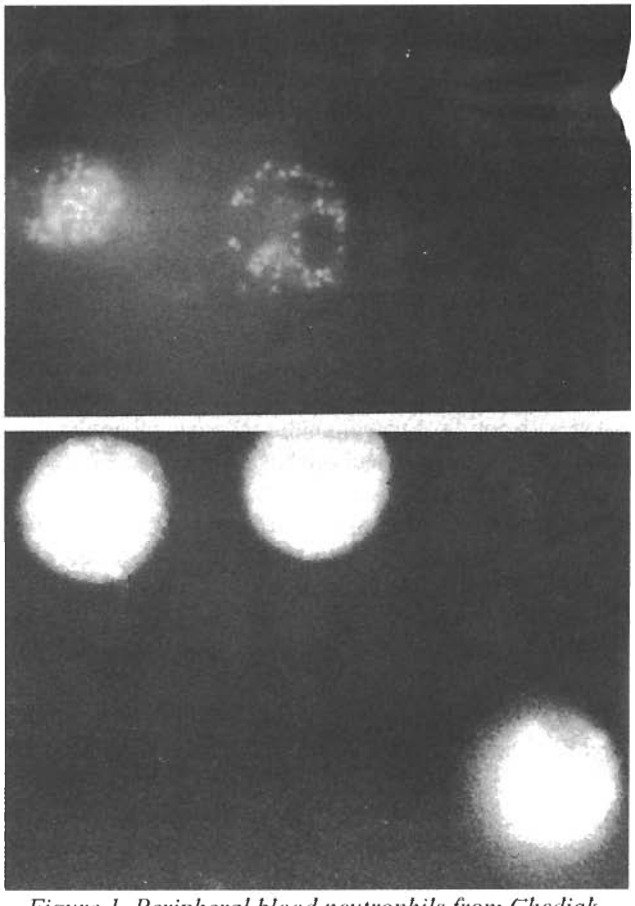

Figure 1. Peripheral blood neutrophils from Chediak-Higashi patient (lower) and control subject (upper) stained with anti-tirosinated tubulin antibodies 1d showing a cytoplasmic granule association in normal cells and a diffuse or non associated patten in CHS neutrophils.

Table 2. Kinetics of cell membrane receptors in CHS patients

Temperature	Time	Distribution patterns in: Controls	CHS Patients
4°C	2 min	Membrane uniform	Cap
37°C	5 min	Membrane aggregates	Cap
37°C	10 min	Membrane aggregates	Cap
37°C	15 min	Cap	Cap
37°C	30 min	Endocytosis	No endocytosis

somewhat intermediate between other patients and controls: a high percent of cells were already spontaneously capped at 4°C, 100% capping at 37°C.

Con A capping in Colchicine treated control cells (at 4°C/2 min) was similar to that seen in untreated CHS cells: 100% spontaneous cap formation. An opposite effect, no cap formation, was seen when normal cells were treated with Cytochalasine B at 37°C for 30 min. However, in CHS cells cytochalasine B did not change the increased Con A membrane receptor mobility (*Table 3*). When normal cells were treated with sodium azide, a metabolic inhibitor, cap formation at 37°C was inhibited up to 30 min of incubation time; this showing that capping is energy dependent and coupled to cell metabolism. In CHS cells, sodium azide did not have any effect, showing a similar fluorescence pattern to that of untreated cells.

By electrophoretic analysis we tried to determine whether there could be differences in the pattern of CHS

Table 3. Drug effect on the kinetics of cell membrane receptors mobility in CHS patients

Drug	Time	Temperature	Distribution patterns in : Controls	CHS patients
Colchicine	2 min	4°C	Cap	Cap
Sodium azide	30 min	37°C	Membrane uniform	Cap
Cytochalasine	30 min	37°C	Membrane uniform	Cap

neutrophil cell proteins as compared to that of normal cells; specially those of the membrane cytoskeleton. In SDS-PAGE electrophoresis of protein extracts from normal cells, some protein bands were easily visualized, specially one with a molecular weight of approximately 50-55 kDa. This protein in immunotransference studies was identified as alpha-tubulin and tyrosinated tubulin. The second peptide detected was of low molecular weight, 43 kDa (a molecular weight similar to that of actin monomers). Some high molecular weight proteins were also seen. The electrophoretic pattern was rather similar in all control subjects studied.

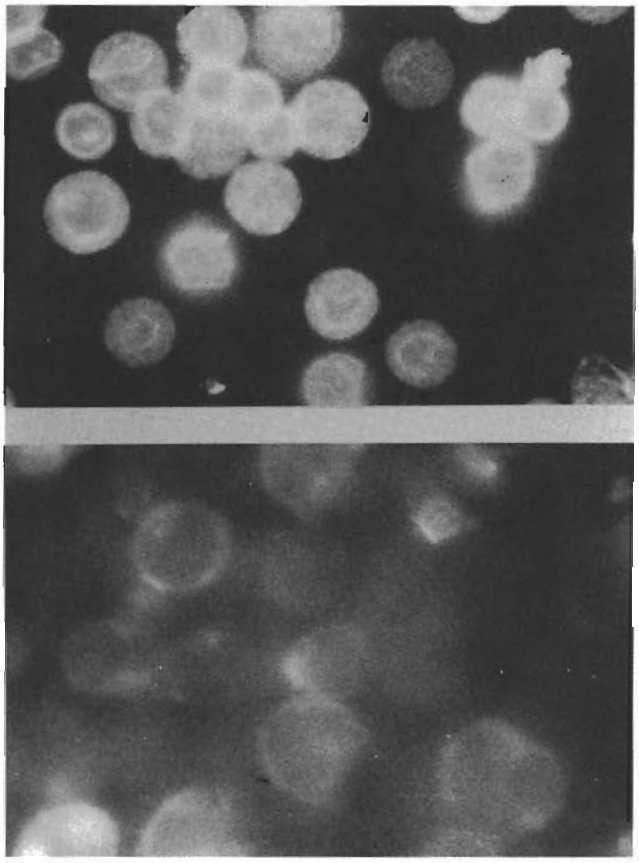

Figure 2. Peripheral blood mononuclear cells from control subject (upper) and Chediak-Higashi patients (lower) stained with anti band 3 antibodies and showing a different degree of fluorescence staining intensity.

Since neutropenia is a common feature in Chediak-Higashi syndrome patients, a rather low amount of proteins is extracted. Therefore, the study of CHS neutrophil proteins presents great technical difficulties. To overcome this problem, we used the silver nitrate staining instead of Coomasie Blue for protein detection in gels. This technique allows detection of quantities in the order of nanograms and enhances visualization of small peptides. Despite these difficulties, it could be recognized that in the gels from CHS neutrophil proteins, the 50-55 kDa protein was very faintly stained as compared to controls (even though similar amounts of proteins were used) (*Figure 4*). This suggests that tyrosinated tubulin was decreased in CHS cells.

DISCUSSION

Chediak-Higashi syndrome (CHS) is a rare autosomal recessive disorder affecting cellular membranes and characterized by the formation of giant organelles in many different cell types. However, only cells such as, neutrophils, melanocytes, CTL populations, NK cells, macrophages and platelets, are both, morphologically affected and functionally defective. Cells such as fibroblasts, mast cells, renal and acinar pancreatic cells are estructurally but not functionally influenced.

The basic cell and molecular defect caused by the CHS gene remains to be fully established. Recent evidence in the literature has indicated that CHS cells afected by the CHS gene show: a) a defective vesicular transport to and from lysosomes and late endosomes [21]; b) aberrant comparmentalization of lysosome and granular enzymes and abnormal trafficking of some cellular proteins [22]; and c) an inability to secrete the protein content of their giant granules: defective degranulation by neutrophils, NK cells and CTL clones [7,8,23].

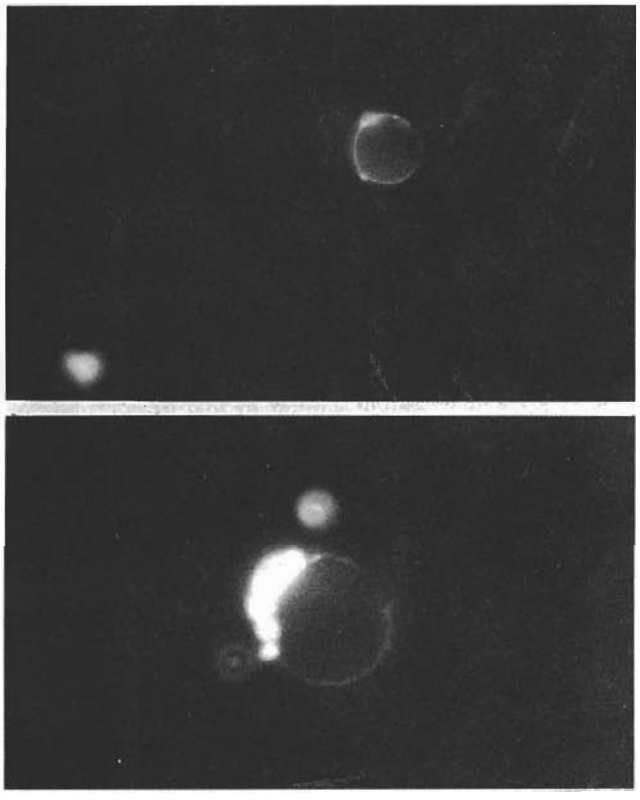

Figure 3. Peripheral blood mononuclear cells from control subjects (upper) and Chediak-Higashi patients (lower) incubated with concanavalin A-FITC at 4°C for 2 minutes, and showing a diffuse staining membrane immunofluorescence pattern with sparse aggregates in control cells and spontaneous capping formation under similar condition by CHS lymphocytes.

The formation of the typical large organelles by CHS cells has been hypothesized to be produced by an increased or abnormal cytoplasmic granular aggregation and fusion. This, can be the result of changes in membrane fluidity [24]; to the inability to secrete their giant granules content due to an altered protein involved in membrane fusion [23]; or to an altered protein involved in vesicle transport and distribution [21].

The CHS gene was associated [25] and has recently been mapped to chromosome 1 (1q42-44) [26-29], and it has been postulated that this gene may define a novel gene family [29]. Even though, the gene product still remains elusive, several hypothesis have been advanced: relationship with proteins asociated with vesicle transport; homology to stathmin, a phosphoprotein that regulates the polymerization of microtubules; and its role as a component of a membrane-associated signal transduction complex that regulates intracellular protein trafficking.

The results presented in this paper indicated that CHS lymphocytes and neutrophils exhibited differences in the celular, cytoplasmic, distribution of some cytoskeleton proteins, and a decrease in the anionic band 3 protein. These results seem to be similar to previously described observations in the literature. Perinuclear acumulations of tyrosinase, TRP-1, and granulophysin, and excessive cellular translocation of tyrosinase-containing vesicles have been described in cultured melanocytes [22] and interpreted as been due to an abnormal trafficking of some cellular proteins. Platelet membrane glycoprotein abnormalities [30], as well as a decrease of some transmembrane proteins such as the Na^+/K^+ ATPase in Chediak-Higashi erythrocyte membranes [31] and the Ca^{2+} pump in beige mouse lysosomes membranes [7] have also been reported. The herein described observations can, therefore, be attributed to abnormal protein trafficking since it is well know that the attachment of these integral membrane proteins to the cytoskeleton may also involve protein sorting. These observations are also compatible with the postulated role of the CHS putative gene product in the regulation of intracellular protein trafficking [29].

As has been previously described [5,6], an increased spontaneous concanavalin A cap formation similar to that of colchicine treated cells was observed. Cytochalasin B, a drug which interferes with actin filament polymerization by specifically interacting with their ends, inhibited cap formation by normal cells, but did not modify the spontaneous capping observed in CHS patients. Interestingly, an *"atypical"* CHS patient because of her age, 19 years old, (most CHS patients perish at about 6 years of age) showed what could be called an "intermediate type" of receptor membrane mobility, even

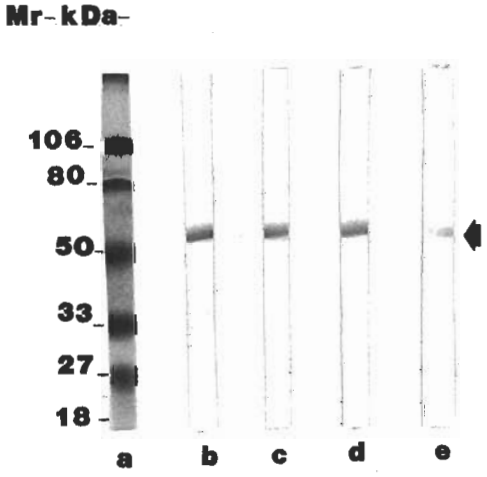

Figure 4. Immunotransference reaction with anti-tyroinated tubulin antibody in (b) control subject, (c) CHS heterozygous, (d) 19 years old CHS patient and (e) CHS patient. (a) molecular weight markers.

though spontaneous caps were produced in about 40% of the cells when incubated with Con A-FITC at 4°C, and the remainder cells showed membrane patches similar to control cells, 100% caps were formed upon incubation at 37°C. This observation seems to indicate that some CHS patients may be less severely affected bt the anomalous gene, probably due to a less gene penetrance.

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