Non-A, Non-B Hepatitis: Ultrastructural Evidence for Two Agents in Experimentally Infected Chimpanzees

Abstract. *Two different ultrastructural alterations were observed in liver cells of chimpanzees inoculated with plasma derived from two different patients with non-A, non-B hepatitis. During the acute phase of illness in one group of four chimpanzees, peculiar tubular structures, composed of two unit membranes with electron-opaque material in between, were observed in the cytoplasm of hepatocytes. In contrast, these structures were never detected in the liver cells of the second group of five chimpanzees that received the second inoculum. However, nuclear changes, usually associated with aggregates of 20- to 27-nanometer particles, were found in hepatocytes of the latter animals. Although these particles resembled viruses, they were not as uniform as small virus particles often appear. In five other chimpanzees inoculated with non-A, non-B hepatitis material not known to be related to the first two inocula, cytoplasmic structures were found in four, and nuclear structures were found in the remaining one. Thus, all 14 chimpanzees inoculated with transmissible non-A, non-B hepatitis agents could be classified as having either nuclear or cytoplasmic changes. These observations add support to epidemiologic data suggesting that there may be more than one agent of non-A, non-B hepatitis.*

Non-A, non-B hepatitis, a disease in humans with characteristics of viral hepatitis, can be diagnosed only by the specific exclusion of viral hepatitis types A and B. The majority of these cases do not appear to be due to other known viral agents such as cytomegalovirus or Epstein-Barr virus. Variability in the clinical and epidemiological characteristics of non-A, non-B hepatitis, as well as reports of multiple attacks of non-A, non-B hepatitis (*1*), have led to speculation that there is more than one etiologic agent.

Hepatitis has been successfully produced (*2*) in chimpanzees by inoculating them with plasma or serum from patients with non-A, non-B hepatitis, thus demonstrating the transmissible nature of the disease. These studies have been confirmed in several other laboratories. In our laboratory, chimpanzees were inoculated with potentially infectious material from a variety of sources, and non-A, non-B hepatitis was produced by at least ten inocula (*2a*). Hepatitis was defined by alanine aminotransferase (ALT) levels in the serum greater than three times the upper limit found in normal serum (40 international units) or by histopathologic changes consistent with viral hepatitis, or both. Since all of these inocula could not be studied with the limited number of animals available, we decided to characterize two of them, chosen because they produced unequivocal evidence of hepatitis and because the illnesses had some distinguishing characteristics that suggested they were caused by different agents. We report here preliminary electron microscopic findings which further suggest the presence of different etiologic agents in these two inocula.

Four chimpanzees were inoculated intravenously with chimpanzee plasma containing an agent (strain F) originally derived from a patient with chronic non-A, non-B posttransfusion hepatitis (*2*) and passaged twice in chimpanzees. All four chimpanzees developed hepatitis, with a mean incubation period to peak ALT level of 11.3 (range 8 to 13) weeks.

Two chimpanzees were inoculated with 10^0 and 10^{-2} dilutions, respectively, of a second agent (strain H), contained in a plasma obtained directly from a patient with acute non-A, non-B hepatitis (*2*). Both animals developed hepatitis, with incubation periods of 6 and 7 weeks. In addition, we studied three other animals inoculated with strain H that did not develop ALT elevations..

Liver biopsies were obtained from these chimpanzees prior to inoculation and weekly thereafter until convalescence and prepared for electron microscopy (*3*). Briefly, liver tissue was fixed in glutaraldehyde, postfixed in osmium tetroxide, treated with uranyl acetate, dehydrated, infiltrated with propylene oxide, and embedded in Epon 812.

During the time of ALT elevation, we noted cytoplasmic alterations in hepatocytes from all four chimpanzees inoculated with strain F. We found peculiar cytoplasmic structures in the cisternae of dilated rough endoplasmic reticulum (ER) (Fig. 1). These cytoplasmic structures appeared circular in cross section and composed of two parallel walls when cut longitudinally. Therefore we believe the structures to be tubular. The walls of the tubules appeared to be constructed of double-unit membranes with electron-opaque material in between. The total thickness of the wall was 20 to 25 nm. The ER was contiguous with both the outer and inner membranes. Occasionally the walls were noted to have two double-unit membranes, but in this case ER was not seen inside the tubule. The diameter of the tubules ranged between 150 and 300 nm and the longest tubule observed was 2.2 μm. The nuclei of hepatocytes from chimpanzees inoculated with the F strain appeared to be normal.

The appearance of the cytoplasmic structures was temporally associated with hepatitis as determined by elevated ALT levels in serum and histopathologic changes characteristic of viral hepatitis in liver biopsies (*4*). We did not detect the structures in liver biopsies collected prior to inoculation, early in the incubation period, or during convalescence.

In contrast, these cytoplasmic structures were not seen in the hepatocytes of chimpanzees inoculated with strain H, but the hepatocytes of all these animals contained distinct nuclear changes. At the time of enzyme elevation, nuclei of the hepatocytes appeared to be heterogeneous in density, condensed, and irregular in shape. Most striking was the appearance of intranuclear aggregates of particles found in four of the five chimpanzees (Fig. 2). The particles measured 20 to 27 nm in diameter. Although they superficially resembled viruses, they were not as uniform as small virus particles often appear. In the cytoplasm, mitochondrial cristae were dilated and ER was distorted.

Table 1. Structures in chimpanzee livers associated with non-A, non-B hepatitis.

Inoculum	Cytoplasmic structures (number positive/ number examined)	Nuclear particles (number positive/ number examined)
Non-A, non-B hepatitis		
Strain F	4/4	0/4
Strain H	0/5	4/5*
Others	4/5	1/5
Hepatitis A	0/3	0/3
Hepatitis B	0/4†	0/4†

*Nuclear particles were not found in one animal inoculated with strain H, but the other characteristic nuclear changes were seen. †Cytoplasmic and nuclear structures characteristic of type B hepatitis were found in hepatocytes of two chronically infected chimpanzees but not in animals with acute infections. These structures were morphologically distinct from those seen in non-A, non-B hepatitis.

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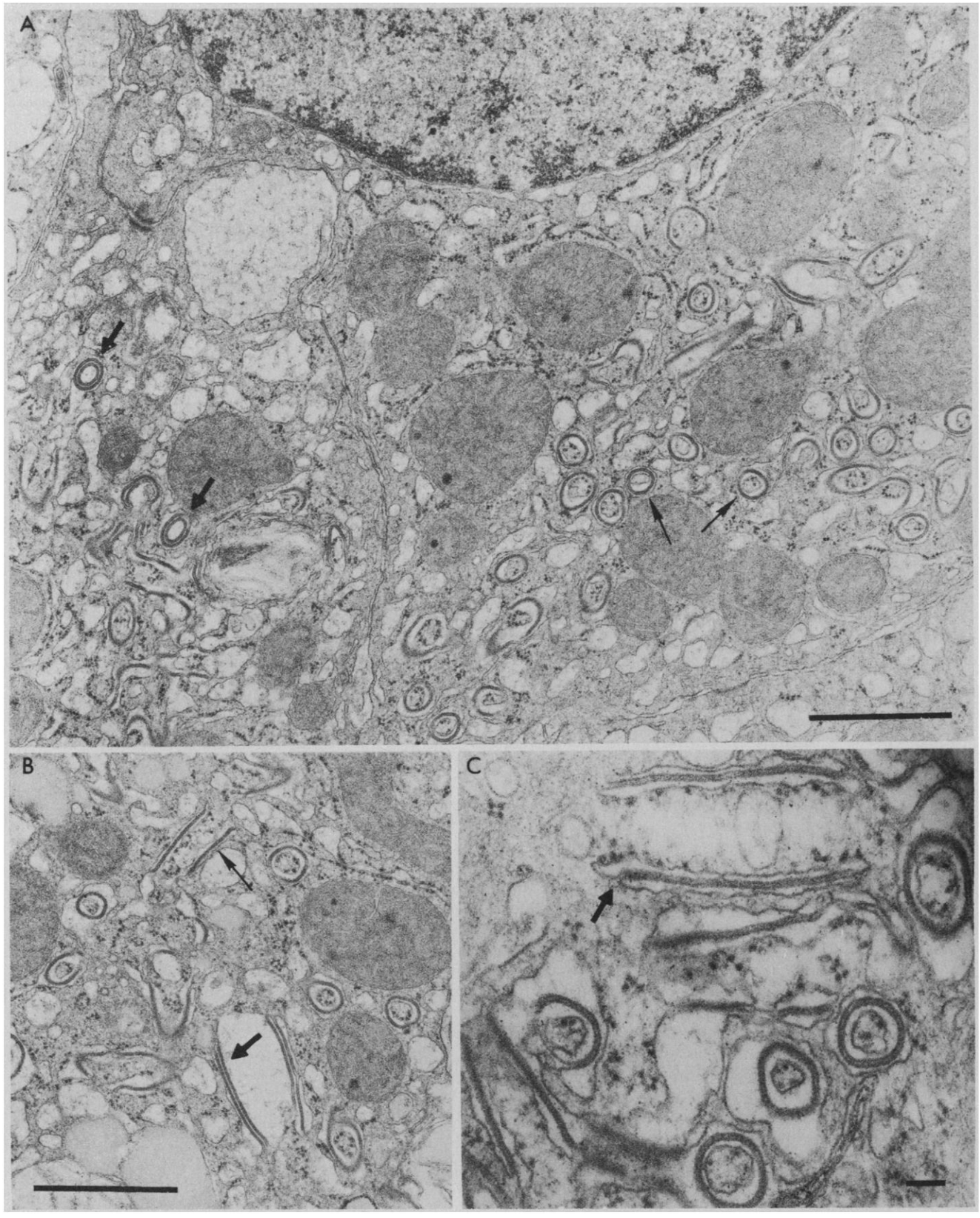

Fig. 1. A hepatocyte from a chimpanzee with non-A, non-B hepatitis 12 weeks after inoculation with strain F, when ALT elevation was maximal. (A and B) Note cytoplasmic structures in the cisternae of ER.These structures appeared circular in cross section [thin arrow in (a)] and as two parallel walls when cut longitudinally [thin arrow in (B)]. Occasionally, the tubular structures were noted to have double walls (broad arrows). The nucleus appeared to be normal. The bar represents 1 μm. (C) Higher magnification of the tubular structures. The walls of the tubular structures appeared to be constructed of double-unit membranes with electron-opaque material in between and a total thickness of 20 to 25 nm. The ER was contiguous with both the outer and inner membranes (arrow). The bar represents 100 nm.

In the chimpanzees inoculated with strain F, only the cytoplasmic structures were seen, while in the chimpanzees inoculated with strain H, only the nuclear structures were seen. The cytoplasmic structures appeared in almost all hepatocytes at the time of peak ALT elevation. In contrast the nuclear particulate structures were not encountered in all cells in the same tissue block nor could they be found in all blocks obtained from the same biopsy. No particles were found in

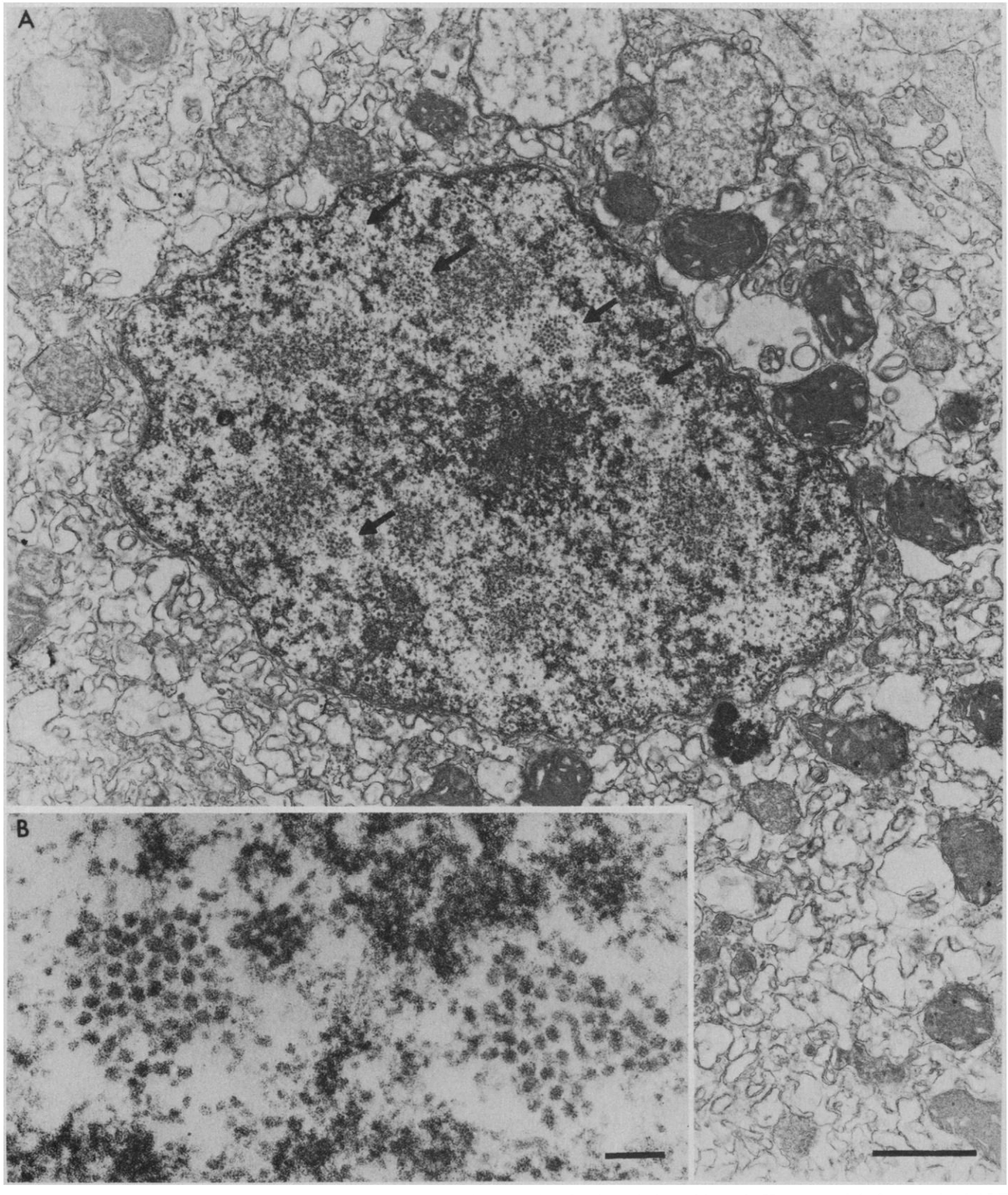

Fig. 2. A hepatocyte from a chimpanzee with non-A, non-B hepatitis 5 weeks after inoculation with strain H, one week before maximal ALT elevation. (A). Distinct nuclear changes are seen. The nucleus is heterogeneous in density, condensed, and irregular in shape. Most striking is the appearance of intranuclear aggregates of particles (arrows). In the cytoplasm, mitochrondria have dilated cristae and ER is distorted. The bar represents 1 μm. (B) Higher magnification of aggregates of intranuclear particles. The particles measured 20 to 27 nm in diameter. Although they resemble viruses, they are not as uniform as small virus particles often appear. The bar represents 100 nm.

one animal that had minimal evidence of hepatitis. However, the other nuclear alterations (irregularity in shape and so forth) were pronounced and seen in almost every hepatocyte.

We also examined five other chimpanzees that had non-A, non-B hepatitis after inoculation with infectious material from a variety of sources not specifically related to strains F or H. These included commercial antihemophilic factor concentrates and plasma from patients with non-A, non-B hepatitis. In addition we studied three chimpanzees with acute hepatitis type A and two chimpanzees with acute and two with chronic hepatitis type B infections (Table 1). We found the cytoplasmic structures in four and the nuclear structures in one of the five chimpanzees with non-A, non-B hepatitis. Neither structure was detected in the livers of the chimpanzees with hepatitis types A or B. Cytoplasmic and nuclear structures were never seen in biopsies from the same animal.

Additional evidence that the cytoplasmic and nuclear changes were specific for non-A, non-B hepatitis was obtained by reexamining selected biopsies under code. Coded biopsies were correctly interpreted as having been taken from an animal inoculated with strain F (cytoplasmic structures detected), strain H (nuclear changes detected), or normal, in the case of preinoculation or convalescent phase biopsies.

In chimpanzees chronically infected with hepatitis B virus (but not those acutely infected), we found structures in the cisternae of ER and core particles in nuclei of hepatocytes characteristic of hepatitis B virus infection. These structures were morphologically distinct from the cytoplasmic and nuclear structures associated with the non-A, non-B hepatitis we describe here (*5*).

Recently, we reported the detection of 24- to 27-nm viruslike particles observed in the cytoplasm of liver cells from a marmoset infected with hepatitis A virus. These particles were shown to contain hepatitis A antigen by peroxidase immunoelectron microscopy (*3*). Similar hepatitis A antigen was detected by peroxidase immunoelectron microscopy in the cytoplasm of hepatocytes from a chimpanzee infected with hepatitis A virus. However, hepatitis A virus particles could not be detected by standard thin-section electron microscopy in the hepatocytes of the three acutely infected chimpanzees studied herein. Nuclear changes were not seen in such hepatocytes. In contrast, Schaffner *et al.* (*6*), demonstrated clusters of dense heterochromatin-like granules, 35 to 40 nm in diameter, in nuclei of hepatocytes of chimpanzees acutely infected with hepatitis A virus. The nuclear structures reported here are morphologically different and have a smaller diameter.

We believe that the cytoplasmic and nuclear structures described herein are specifically associated with non-A, non-B hepatitis and that they may be of significance as markers of infection, although we cannot exclude the possibility that they might represent hitherto unknown nonspecific responses of hepatocytes to injury. The relationship of the structures to the specific infectious agent or agents of non-A, non-B hepatitis remains uncertain. Specificity will have to be shown by some means such as immunoelectron microscopy. To date, attempts to identify an antigen-antibody system for non-A, non-B hepatitis have been unsuccessful.

The possibility that there may be more than one infectious agent as the cause of non-A, non-B hepatitis is suggested by a report of multiple bouts of non-A, non-B hepatitis in patients, whose biopsies showed evidence of acute infections for each bout (*1*). The present observations of different morphological changes at the electron microscopic level produced by different inocula add support to the earlier data suggesting the possibility of more than one non-A, non-B hepatitis agent. Furthermore the finding that every transmissible agent examined by us produced one or the other morphological change offers hope that the total number of non-A, non-B virus types will be small.

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Erythrocytes: A New Cell Type for the Evaluation of Insulin Receptor Defects in Diabetic Humans

Abstract. *Human erythrocytes have specific insulin receptors. When studied in an insulin radioreceptor assay, erythrocytes from adult-onset, nonobese diabetic subjects bound at least 42 percent less insulin than the normal subjects at insulin concentrations from 0.1 to 100 nanograms per milliliter. The diabetic subjects had 190 insulin receptor sites per cell as compared with the 380 insulin receptor sites per cell for the normal subjects. The deficit of insulin binding in the diabetic subject was thus associated with a fewer number of insulin binding sites per cell with little or no change in affinity. The erythrocyte is a readily available cell for the evaluation of cellular insulin receptor activity.*

Insulin action in humans has been studied at the cell receptor level in obesity (*1–6*), diabetes mellitus (*6–10*), pregnancy (*10*), uremia (*11*), acromegaly (*12*), ataxia telangiectasia (*13*), and the syndrome of severe insulin resistance with acanthosis nigricans (*14*). Insulin receptors have been defined in human mononuclear cells (*1–3, 5, 7, 8, 13–16*), granulocytes (*17*), adipocytes (*3, 4, 6, 18*), placental cells (*10*), and cultured fibroblasts (*19, 20*). Intracellular binding sites have been identified in nuclei (*21*), on the Golgi apparatus (*22*). These studies of insulin receptors have been done with cells that, although accessible, are obtained and isolated only after time-consuming processes. The mature human erythrocyte is easily obtained, easily isolated, and is the most abundant circulating cell.

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