In Vitro Morphological Studies on Antibody-Dependent Nonimmune Lymphocyte-Mediated Cytotoxicity in Chronic Active Liver Disease

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Using an in vitro *system of antibody-dependent cellular cytotoxicity (ADCC), the killing effect of chronic liver disease sera on target Chang cells, mediated by effector nonimmune lymphocytes (NLy), was studied. NLy destroyed Chang cells in monolayers pretreated with sera of patients with chronic active liver disease (CALD). Sera from these patients with CALD, after receiving steroid therapy, demonstrated a significant decrease of the cytotoxic action of NLy. The target cells treated with sera of normal subjects or patients with chronic persistent hepatitis were only minimally affected. Morphological observations of the cytotoxic action in a CALD serum-treated group showed intimate contact between NLy and the target cells in the areas of the plaques, where large numbers of the target Chang cells were injured and were closely associated with effector NLy. The Chang cells developed cytoplasmic swelling. The surface became ruffled, and intracytoplasmic organelles displayed vesicular degeneration. Thereafter, cell rupture and fragmentation occurred. The sera in patients with CALD appear to possess a membrane reactive factor, presumably antibody, against the surface membrane of Chang cells. This immunological mode of reaction between the effectors and target cells (ADCC) may be important in the perpetuation and pathogenesis of hepatocyte death in CALD.*

There is evidence suggesting the participation of both cellular and humoral immune processes in the liver of patients with chronic active liver disease (CALD) (1–12). The autoimmune phenomena seem to be responsible for hepatocyte injury in that primary immune reactivity appears to be directed against liver tissue constituents in the presence of specific autoantibodies. Liver-specific protein antigens, associated with the cell membrane of hepatocytes (13) and cytoplasmic organelles (13, 14), have been characterized and, using some of these antigens, autoimmune chronic active hepatitis has been produced in rabbits (6). Self-directed antibodies, such as anti-liver-specific lipoprotein antibodies (2), and liver-specific antigens, such as F-antigen (14), are often present in the sera of patients with CALD. In our previous study (11), using liver biopsy specimens from patients with chronic liver disease, the close morphological association of lymphocytes with hepatocyte injury was found in CALD.

One possible mechanism for the induction of target liver parenchymal cell injury or death in CALD, involving both cell-mediated and humoral immune systems, is antibody-dependent cellular cytotoxicity (ADCC), which has been extensively

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studied in various animal and other human experimental models (15–26). This immune mechanism is characterized by the killing of antibody-coated target cells by NLy. No complement is required for this target cell lysis.

The present study is, therefore, an attempt to investigate an *in vitro* ADCC in the immune pathogenesis of CALD. We have particularly focused on demonstrating similar morphological interrelation *in vitro*, as seen in liver tissues of patients with CALD, between effector lymphocytes and target Chang cells sensitized by CALD sera, which presumably contain anti-liver cell-surface antibody.

MATERIALS AND METHODS

Sera. All aera studied were obtained from 10 normal healthy individuals, 7 patients with chronic persistent hepatitis (CPH), and 9 patients with chronic active liver disease (CALD) before prednisone therapy. Post-steroid-treated sera of the patients with CALD were also used. These specimens were inactivated at 56° C for 30 min, followed by absorption with minced and lyophilized human cadaveric kidney tissues (less than 6 hr postmortem) at 37° C for 60 min and subsequently overnight at 4° C. The treated test and control sera were then stored at −20° C before use. The diagnosis of patients with chronic liver disease (CPH and CALD) were established on the basis of clinical, biochemical, and histological data (27). The CALD patients, treated with prednisone, were all in good clinical and biochemical control, as assessed by the criteria of the Mayo group (27).

Target Cells. The target cells used in this study were a human liver cell strain of Chang, purchased from Microbiological Associates, Bethesda, Maryland.

Effector Lymphocytes. Venous peripheral lymphocytes from normal individuals ranging from 18 to 41 years of age were separated by dextran and Ficoll-Hypaque gradients (28). Contaminating monocytes were removed by adhesiveness to plastic tissue culture wells in a moisturized atmosphere (95% air–5% CO_2) at 37° C for 2 hr. More than 98% of the recovered cells were lymphocytes, which were differentiated by Wright-stain smears, and peroxidase stain (29) for monocytes. The lymphocytes had greater than 95% viability as assessed by trypan blue dye (0.05%) exclusion. The cells were washed 3 times with Hank's balanced salt solution (HBSS) before being added to the test system.

***In Vitro* ADCC Test System.** The methods used are a modification of those described by Biberfeld et al (30). Approximately 3×10^4 Chang target cells were first grown in a 35 × 10-mm Falcone tissue culture well (#3001) with 2.5 ml of 80% Eagle's Minimal Essential Medium (MEM)–20% heat-inactivated fetal calf serum (56° C, 30 min) (FCS) supplemented with L-glutamine (2 mM/ml) and antibiotics (100 units of penicillin and 100 μg of streptomycin/ml) at 37° C in a moist atmosphere of 95% air and 5% CO_2. The monolayers were obtained after 72 hr incubation. Each culture dish was washed 3 times with HBSS to eliminate dead cells and cell debris. The Chang cells in monolayer were treated with 1 ml of each test or control serum, diluted to 10^{-2} with Eagle's MEM, or 1 ml of the latter nutrient medium alone, for 60 min under the above conditions. Our previous observation by immunofluorescence showed that membrane-fixed IgG in the sera from patients with CALD was weakly detected on Chang cells up to a dilution of 10^{-2} (31). All CALD sera tested contained anti-human and -rabbit hepatocyte surface membrane antibodies (IgG), which were shown also by immunofluorescence (32). Thereafter, the culture dishes were thoroughly washed 5 times using HBSS. Culture medium, 1.5 ml, consisting of 90% Eagle's MEM–10% FCS with L-glutamine and the antibiotics, was added to each well. Then 2×10^6 lymphocytes in 0.05 ml of the culture medium were placed over the central part of the monolayers. Approximate estimation of a ratio of the effector cell to target cell was at 10:1 within the plaque area (33).

The cultures were maintained in water-saturated 5% CO_2 in air at 37° C for 72 hr. Each experiment was performed in duplicate. At the termination of each culture, the following studies were carried out:

LDH (Lactic Dehydrogenase) Determination. LDH activity of the supernatant from each culture medium was measured, using a centrifugal analyzer (LDH-L, Smith Kline Instruments, Inc., Palo Alto, California) (34).

The Wilcoxon rank sum test was used for statistical analysis.

Plaque Formation (PF). PF in the central areas of the monolayers, treated as described, was estimated according to the scale shown in Figure 1.

Morphological Observations. Target cell killing by NLy was followed at daily intervals with a phase-contrast inverted microscope. The cultures of ADCC test systems were also made on 2.8-mm round coverslips in quadruplicate. The duplicate coverslips were fixed in 95% alcohol, and stained with Wright's solution. For scanning electron microscopy, the cells grown on the remaining coverslips (duplicate) were rinsed twice with phosphate-buffered saline solution (PBS) (pH7.4), and then fixed, using 2% glutaraldehyde in 0.1 M cacodylate buffer. The fixed specimens were dehydrated through graded ethanol, followed by 2 washes of 100% amyl acetate and CO_2 critical-point drying (35). After coating with gold of approximately 100 Å, they were examined in a JSM-U3 scanning electron microscope at a beam voltage of 20 kV. For transmission electron microscopy, the cultured cells were fixed *in situ* with the cacodylate-buffered glutaraldehyde. The culture media were used for determination of the activity of LDH (see above). After postfixation in 1% O_8O_4 and dehydration, the cells were detached from the dishes (36) and embedded in Epon. Sections 1 μm thick stained with 1% toluidine blue were used for orientation. Thin sections of the specimens were examined in a Hitachi HU-11C-1 electron microscope.

RESULTS

LDH Assay. Table 1 shows the LDH levels in culture media from the various experiments. There was a significant increase of LDH activity in cultures with NLy and Chang monolayer cells (no se-

TABLE 1. ANTIBODY-DEPENDENT CELLULAR (LYMPHOCYTE) CYTOTOXICITY, ESTIMATED BY LDH ASSAY AND PLAQUE FORMATION (PF)—CULTURES OF 3 DAYS

Experiments				
Types of cells in culture	*Source of serum used for pretreatment of target Chang cells*	*Number of samples*	*LDH* (IU/ml + 1 SD)*	*PF†*
Chang cells	None	10	98.8 ± 8.6	0
Nonimmune lymphocytes (NLy)	None	10	29.5 ± 3.2	0
Chang cells + NLy	None	10	152.6 ± 10.6	0 to 1+
Chang cells	Normal serum	10	104.7 ± 5.0	0
Chang cells + NLy	Normal serum	10	156.5 ± 8.3	0 to 1+
Chang cells	CPH serum	7	102.6 ± 5.9	0
Chang cells + NLy	CPH serum	7	153.0 ± 9.0	0 to 1+
Chang cells	CALD serum	9	104.1 ± 9.3	0
Chang cells + NLy	CALD serum	9	242.8 ± 32.1	2+ to 3+
Chang cells	Steroid-treated CALD	9	106.0 ± 15.4	0
Chang cells + NLy	Steroid-treated CALD	9	172.6 ± 26.1	0 to 2+

*Statistical analysis: see text.
†Scoring of PF (0 to 3+): see Figure 1.

rum treatment) ($P < 0.001$) compared with cultures of either cell type alone. Treatment with control or test sera alone did not induce cytotoxicity in Chang cells. However, LDH activity in the media increased if Chang cells, pretreated with CALD sera, were exposed to NLy ($P < 0.001$). In the steroid-treated patients with CALD, the LDH values decreased ($P < 0.001$), compared with the nontreated patients, but remained greater than those of the N and CPH groups ($0.01 < P < 0.05$).

PF. The degree of central clearance of the monolayers of Chang cells at the sites covered by NLy are shown in Figure 1. Small plaques (H) were often demonstrated in the cultures of NLy with Chang cells, whether non-precoated or precoated with N or CPH sera (Table 1). Enhancement of PF was observed in Chang cells pretreated with CALD sera in the presence of NLy (Table 1). The PF became evident on the second day of incubation and had increased by the third day.

Morphological Interaction between Effector NLy and Target Chang Cells. Examination of unstained phase-contrast and stained specimens indicated close cell-to-cell contact of these two cell types, but only in areas of developing plaques, as shown in Figure 2. There were many rosettes, formed by the effector and target cells (Figure 2b,c). The target cells surrounded by these NLy began to swell and become round with increased intracytoplasmic vesicular inclusions (Figure 2c). Thereafter, the cells became detached. In contrast, most of the attached target cells not exposed to NLy did not display cytopathic changes. Epon-embedded thick survey sections permitted examination of the structural relationships of these effector and target Chang cells by sectioning vertically to the plane of the monolayer (Figure 3a–d). Following exposure of Chang cells to sera from patients with CALD, NLy established contact with the surface of the target cells by many small cell membrane projections (pseudopods) (Figure 3a). NLy often migrated into the intercellular space between two target cells (Figure 3b). The Chang cells adhering to NLy appeared swollen and rounded, while some had ruptured (Figure 3d).

Scanning electron microscopic observation demonstrated that a few small or medium-sized lymphocytes often adhered to one or several elongated cytoplasmic projections of Chang cells pretreated with CALD sera on the culture coverslip (Figure 4b). The surface appearance of NLy was either hairy or smooth (Figure 4b-d). These Chang cells reacting with NLy displayed swollen and rounded cellular contours with many cytoplasmic ruffled buddings on the surface (Figures 4b,c). Microvilli on the surface disappeared as seen in intact Chang cells (Figure 4a). The cell membrane of the degenerating target cells in close association with NLy had disintegrated further and finally fragmented and ruptured (Figure 4d). In transmission electron microscopy, NLy again formed direct contact with the surface microvilli of the CALD serum-treated Chang cells by the minute projections of the effector lymphocytes. These NLy had the appearance

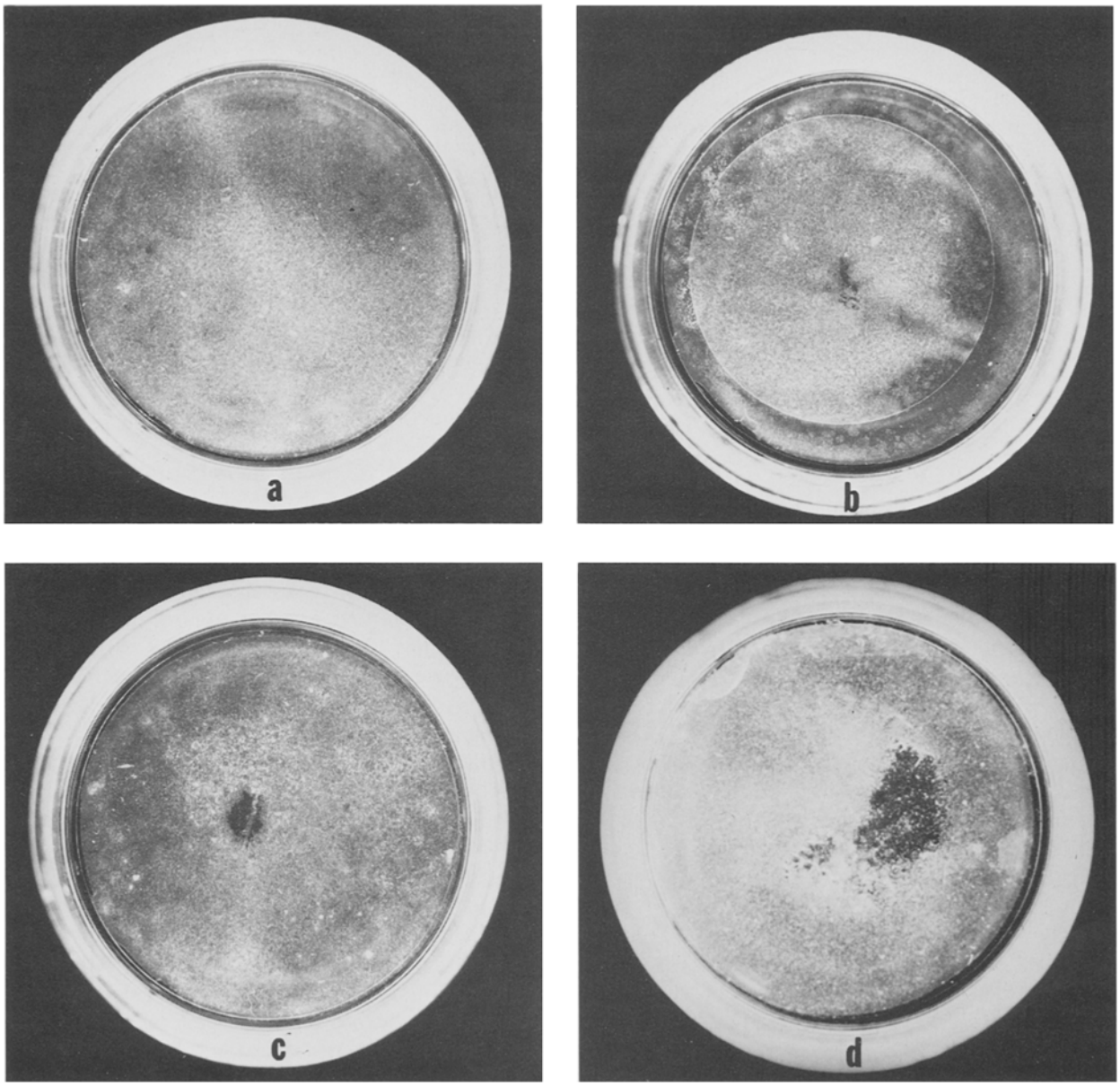
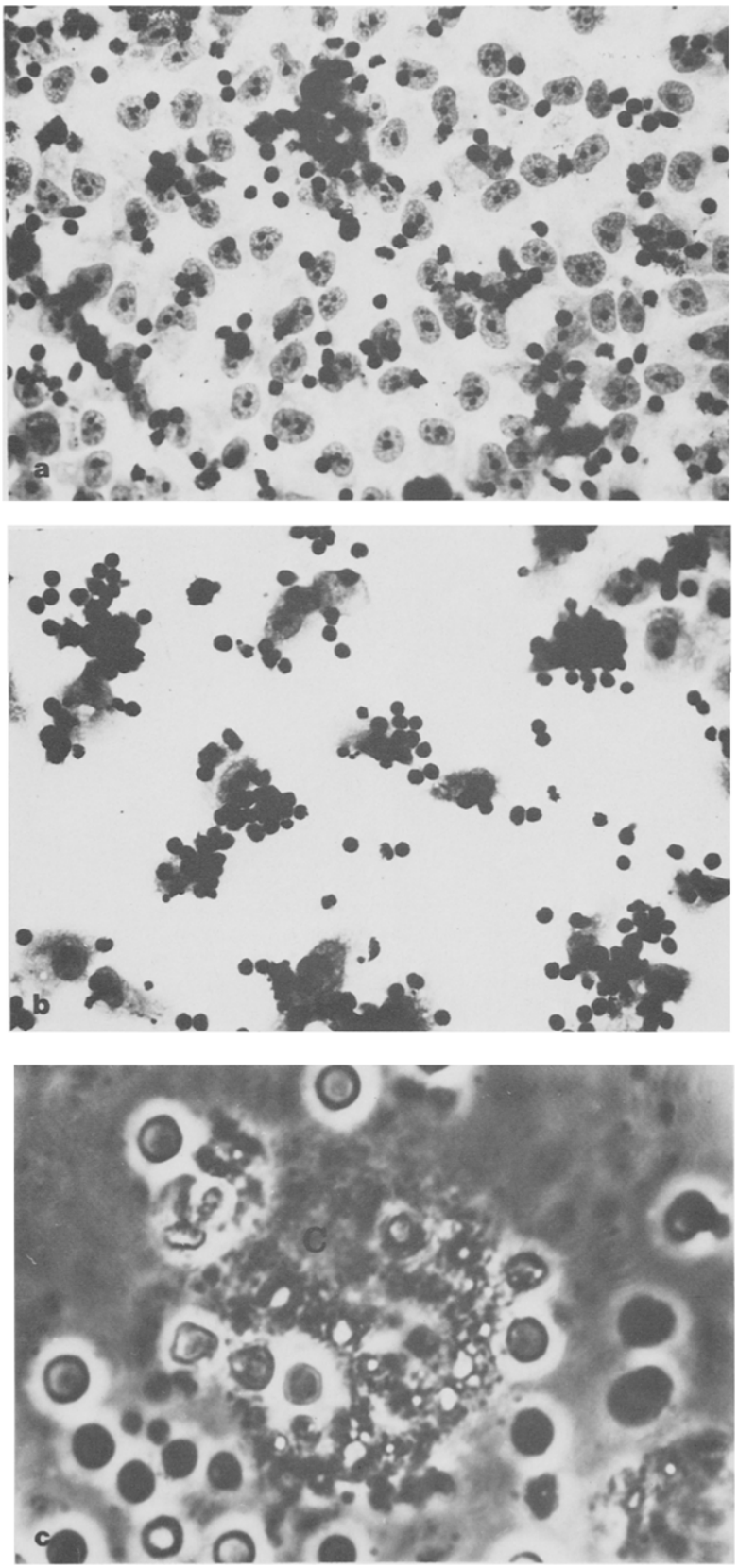


Fig 1. Plaque formation (PF) in monolayers of control or test serum-precoated Chang cells induced by nonimmune lymphocytes (after washing with HBSS): (a) CPH serum-treated culture, (b) normal serum-treated culture, (c) CALD serum-treated culture, and (d) CALD serum-treated culture, demonstrating 0, +, ++, and +++ PF, respectively. Note prominent adherence of lymphocytes in the overlayed central part of culture (d), as seen by a denser zone even after washing.

Fig 2. (facing page) Light micrographs of the interaction between effector nonimmune lymphocytes and Chang cells in the presence of test sera (CPH and CALD): (a) Treatment with CPH serum, not showing cytotoxicity of the target cells (Wright stain, ×350), (b) treatment with CALD serum, showing adherence of lymphocytes to remaining Chang cells at the site of PF (Wright stain, ×340), (c) treatment with CALD serum, displaying a few lymphocytes attaching to a swollen and injured Chang cell (C); note abundant intracytoplasmic vesicular inclusions. (phase contrast, ×1080)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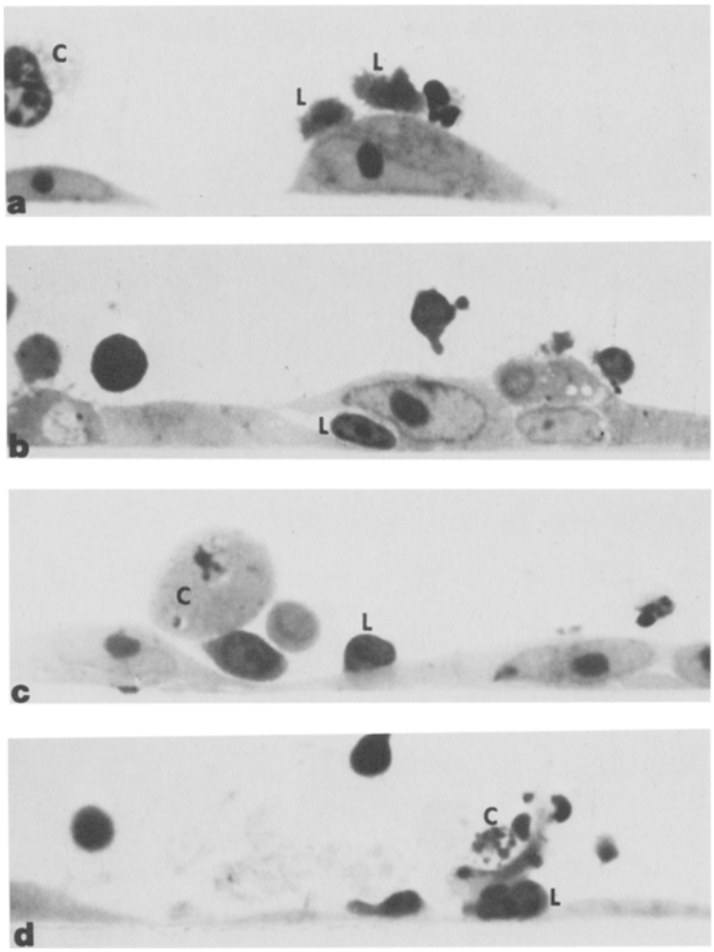

Fig 3. Light micrographs of epon-embedded thick sections of Chang cell monolayers treated with CALD serum: (a) two small lymphocytes (L) contacting a Chang cell by means of multiple cytoplasmic projections of the effectors. A detached swollen Chang cell is seen with marked injury (C). (b) A small lymphocyte (L), infiltrating between two Chang cells. (c) A degenerating rounded Chang cell (C), which appears to be contacted by surface minute processes of a lymphocyte. Note a lymphocyte (L) adhering to a Chang monolayer cell. (d) A rupturing and fragmenting Chang cell (C). A lymphocyte (L) is in contact with the dying cell. (toluidine blue stain, ×1400)

of a nondividing nuclear pattern with well-developed organelles and frequently increased lysosomal elements (Figures 5a,b). There was no morphological evidence of blast transformation of the NLy nor evidence of a NLy entering the Chang cell (emperipolesis) (37). Cytoplasmic swelling, and vesiculation of intracellular organelles, appeared to precede target cell fragmentation. Study of the detached Chang cells in the supernatant indicated that the majority of these cells were severely damaged or dead with cytoplasmic vesicovacuolation (Figure 6).

In cultures incubated without serum or with normal sera or sera from patients with CPH, the above-mentioned observations were much less frequent or absent (Figures 2a, 4a).

DISCUSSION

The *in vitro* destruction of antibody-coated target cells by NLy (ADCC) has been described in many

experimental systems, including cytolysis of red cells (16, 19), allogeneic (22) and autologous lymphocytes (23), allogeneic leukemic cells (24), and various human tissue culture cells (17, 18, 25, 26). This report appears to describe an *in vitro* model for the ADCC of CALD. The elevation of LDH levels and PF in cultures of Chang cells, pretreated with CALD sera in the presence of NLy, correlates with cytological observations of target Chang cell death.

Chang cells, which were used as target cells here, were first introduced in 1954 as an epithelial-like human adult liver cell line (38). This cell line has been utilized for studies of the cellular immune response, including immune cytotoxicity assays in acute and chronic hepatitis (8, 9, 30, 31, 33). However, the validity of use of Chang target cells as models of human hepatocytes has recently been questioned because of similarity of isoenzyme patterns and chromosomal characteristics between Chang cells and HeLa cells (39). Chang cells from the same source (American Type Culture Collection) as those in the latter study, however, have been found to synthesize human albumin, fibrinogen, transferrin, $alpha_2$ macroglobulin, $beta_1$ lipoprotein and C'3 complement (40). HeLa cells not only fail to complete these functions but are also morphologically different from Chang cells (40). Interestingly, lymphocytes from patients with acute and chronic active hepatitis can cause significant lysis of not only autologous human liver cells, but also Chang cells *in vitro* (8, 9). Thus, Chang cells seem to have many characteristics in common with liver parenchymal cells. To verify the use of Chang cells as human hepatocytes in *in vitro* immunological studies an antigen characterization of the surface membrane of the former cells is further required.

In the experiments reported here, NLy alone caused minimal lysis of nontreated Chang cells. This nonspecific cytodestruction may be due to HL-A incompatibility (41) or to spontaneous lymphocyte-mediated cytotoxicity, as is demonstrated in other *in vitro* systems (42).

Cytolysis of CALD serum-treated Chang cells was significantly enhanced by NLy. This finding is in contrast to the lack of enhancement of cytolysis of Chang cells treated with normal or CPH sera. This enhancement of target lysis appears to indicate that the sera from patients with CALD reacts with the surface membrane antigens of the Chang cells and that the antigen–antibody complexes may activate NLy with resultant killing of the target cells (30). It has previously been demonstrated that the CALD sera, absorbed with Chang cells or human adult liver cells failed to induce target Chang cell destruction *in vitro*, determined by a microcytotoxicity assay in the ADCC similar to the present system (31). This evidence seems to suggest the presence of immunological cross-reactivity between Chang cells and human liver cells at least to some extent. However, because of the questionable identity of the Chang target cells as human hepatocytes, the reactive antibody on the surface of Chang cells in the test sera of CALD might be associated with new antigens of modified cell membrane protein of human liver parenchymal cells, produced by hepatitis viruses (10) or unknown hepatotoxic agents. The antibody may, therefore, cross-react between two cell types in certain pathological conditions, like CALD. On the other hand, it cannot be entirely excluded in the present experiments that the toxic effect of normal lymphocytes in the presence of the antibodies on the target cells may be nonspecific, since target cells other than Chang cells were not tested.

The reduction of cytotoxicity by NLy when steroid-treated CALD sera were used in the same patients studied before the treatment may be explained by a decrease of anti-target-cell membrane antibody in the test sera and subsequent inhibition of lymphocyte activation by contact with the target cells (43). This hypothesis will be proved by measuring the antibody titers of each CALD serum tested, which was not done in the present study. Whatever the mechanism is, even in CALD cases receiving steroids, some immune target cytodestruction by ADCC persists.

NLy, adhering to the target Chang cells precoated by CALD sera, display features of active movement, as suggested by infiltration of the Chang cell monolayers. The increased motility of NLy may be caused by their interaction with antigen–antibody complexes on the surface of the target cells (41). The presence of multiple pseudopods in the active NLy may provide further evidence of their functional activation. However, the absence of uropods, frequently present in lymphocytes stimulated by PHA or Con A (15) and in a similar ADCC model *in vitro* (30), suggests that the NLy activation may be less extensive. As in the other reports on ADCC (15–18, 41), the cytotoxic action of NLy does not require complement, and these lymphocytes show neither blast transformation nor phagocytic activity during target cell lysis.

The effector lymphocytes participating in ADCC

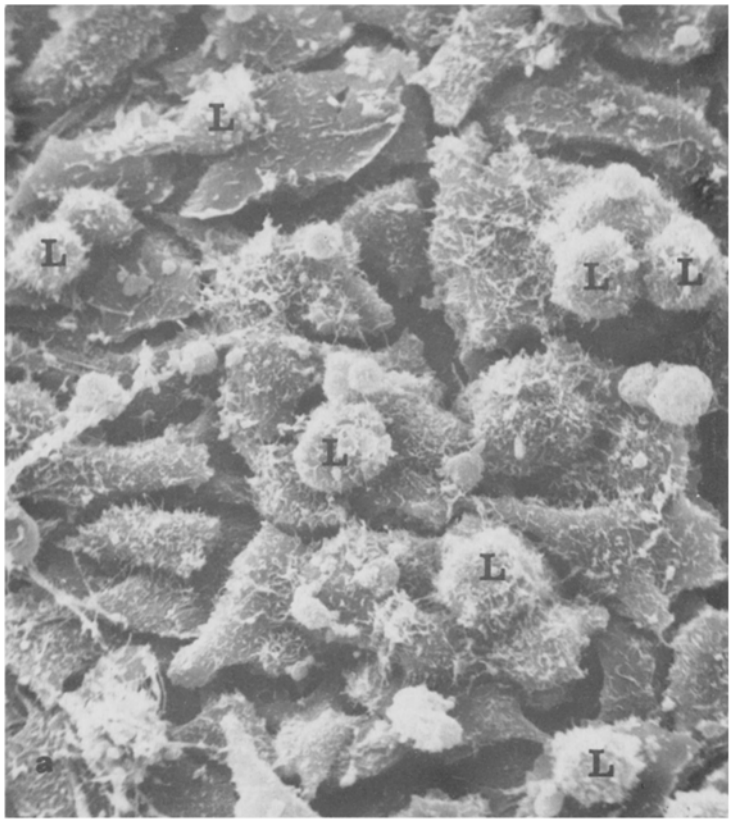

Fig. 4A. Scanning electron micrograph demonstrating cytopathic effect of lymphocytes on Chang cells. A normal serum-precoated Chang cell monolayer with lymphocytes (L). Note no destruction of the Chang cells. (×1300)

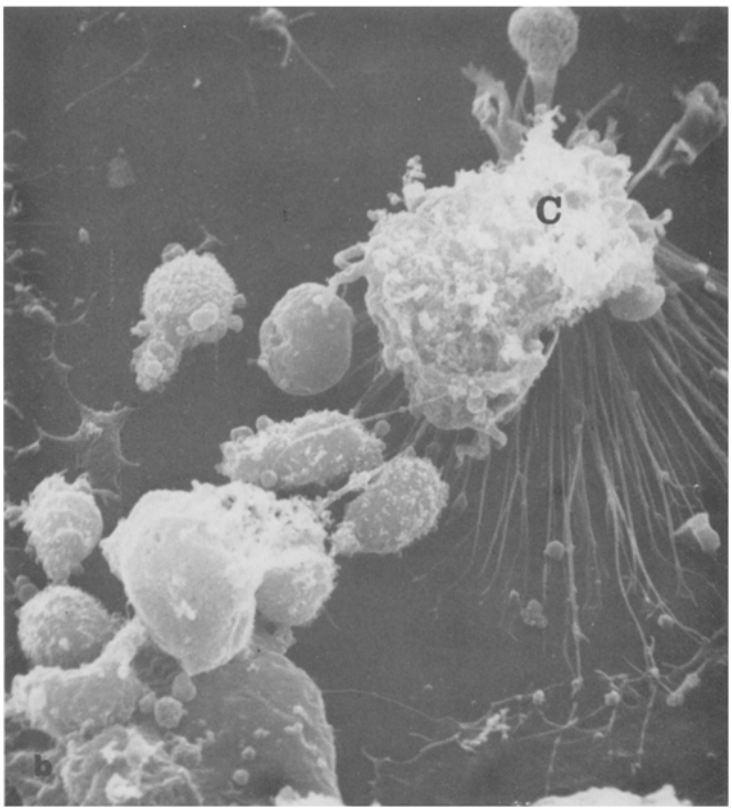

Fig 4B. Scanning electron micrograph demonstrating cytopathic effect of lymphocytes on Chang cells. A CALD serum-treated Chang cell (C), injured by adherence of hairy and smooth-surfaced lymphocytes on long cytoplasmic processes of the former cell. The Chang cells are swollen and rounded in association with ruffled cell surface. (×3250)

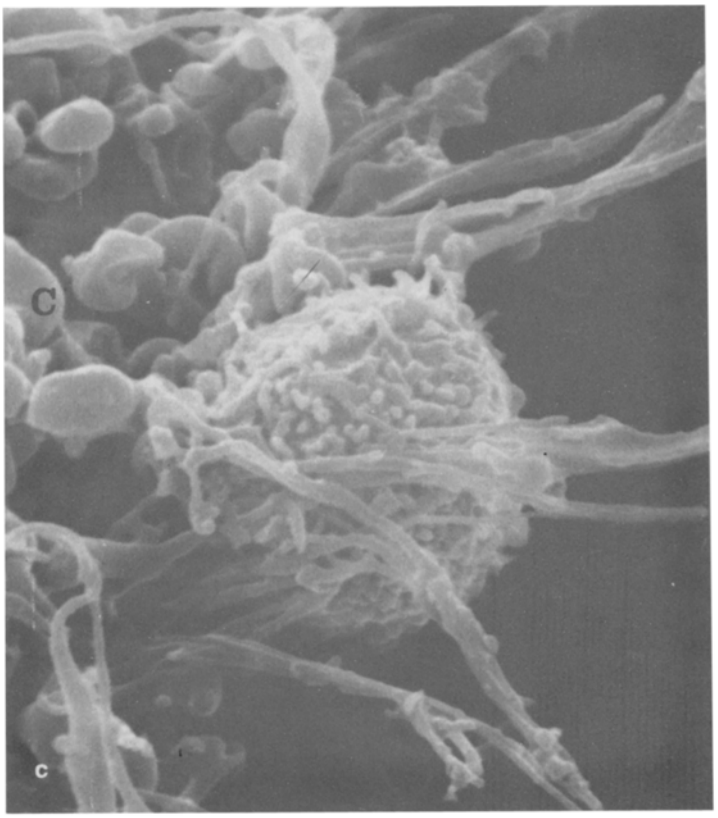

Fig 4C. Scanning electron micrograph demonstrating cytopathic effect of lymphocytes on Chang cells. A CALD serum-treated Chang cell (C) in intimate contact with a hairy lymphocyte, which appears to elevate some of the foot processes of the former cell. The surface of the Chang cell exhibits multiple cytoplasmic budding. (×10,400)

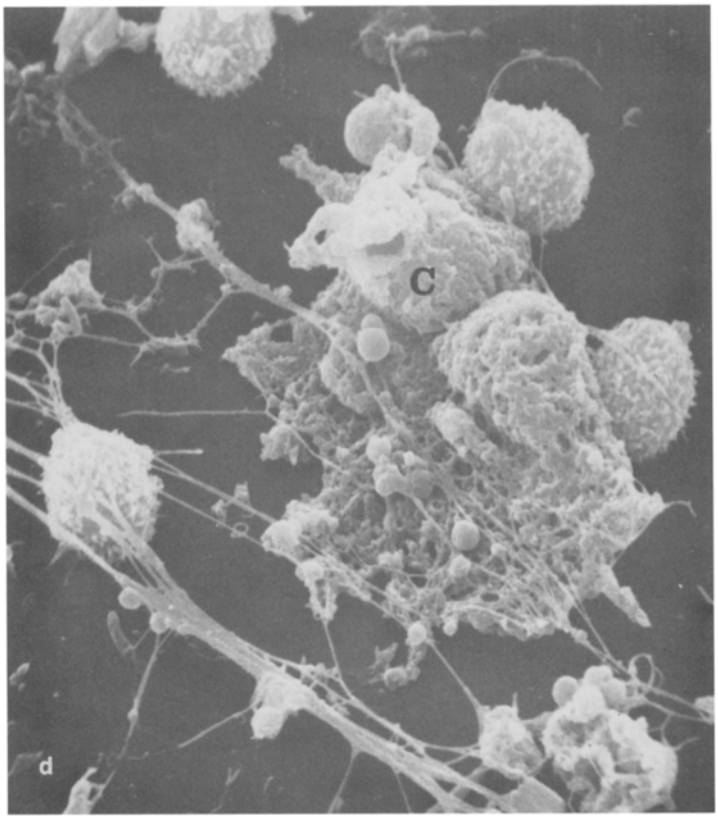

Fig 4D. Scanning electron micrograph demonstrating cytopathic effect of lymphocytes on Chang cells. A markedly damaged and ruptured Chang cell (C), pretreated with CALD serum. Two lymphocytes are still adhering to the target cell. Many fragmented cellular components of various sizes are seen on and around the Chang cell. (×4380)

Fig 5. Transmission electron micrographs of cultures, treated with CALD sera. (a) A small lymphocyte (L) migrating into an intercellular space between two Chang cells. There are well-developed cytoplasmic organelles. Note some increase in lysosomes (ls). The lymphocyte is attached to a Chang cell via microvillous structures of the latter (arrowheads). (×20,400) (b) A small lymphocyte (L) adhering to a Chang cell through surface microprojections of the former cell (arrowheads). (×20,400)

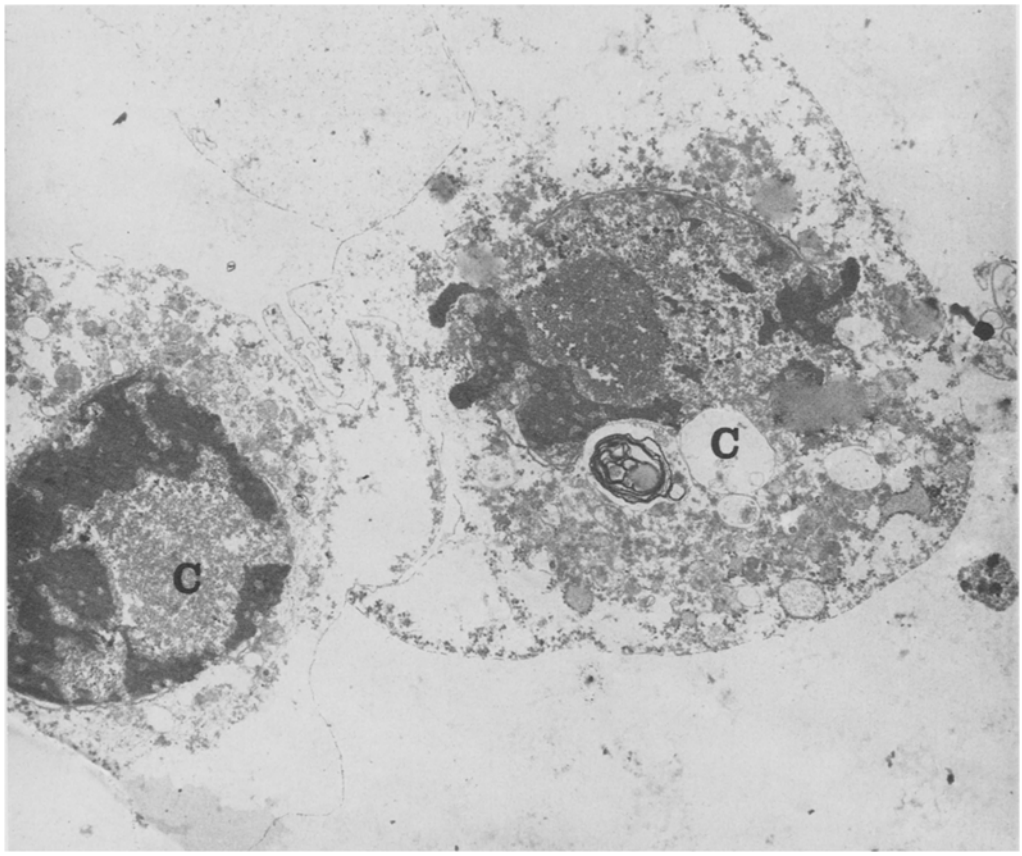

Fig 6. Transmission electron micrograph of two rupturing and swollen dead Chang cells (C) in the supernatant of CALD serum-treated culture medium, suggestive of osmolysis (×8400)

have been characterized as possessing Fc receptors on the surface membrane, lacking the ability to form both sheep red cell rosettes and devoid of detectable membrane Igs (44). At present, it seems likely that the major effectors of NLy in the system of ADCC are null cells and their progeny (20, 21, 23).

The morphological evidence of Chang cell lysis mediated by NLy in the *in vitro* system exhibits great similarity to our previous observation (11), which showed the close association of lymphocytes with hepatocyte death via cell-to-cell contact in the liver of CALD *in vivo*. The present studies suggest that the intimate surface attachment between the effector NLy and target Chang cells pretreated with CALD sera may initiate the cytolysis of the latter cells. The subsequent sequence of destruction of Chang cells (swelling with ruffled surface, then rupture and fragmentation) is similar to that described by Biberfeld et al (30), who demonstrated cytopathological changes in Chang cells in the presence of anti-Chang cell serum when these target cells were exposed to NLy. This was suggested to be due to osmotic lysis. Humoral factors, released from activated lymphocytes at the sites of effector target cell contact, may be involved in the target cell death (30).

In conclusion, our studies show that target Chang cell lysis was initiated by direct contact with effector NLy in the presence of CALD sera, the latter probably containing antibodies against Chang cell-surface membrane antigens. As described above, the antibodies in sera from CALD patients may cross-react with Chang cells and human hepatocytes under certain abnormal conditions. The target cells appear to exhibit an osmotic lysis preceded by

swelling and rupture. The *in vitro* target cytodestruction may be a useful model for the study of hepatocyte injury in CALD.

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