T-Lymphocyte Subpopulations and Function during Murine Cytomegalovirus Infection

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ABSTRACT. To study the effects of cytomegalovirus infection on T-lymphocyte subpopulations, we determined helper (Lyt 1.2) and suppressor (Lyt 2.2) T-lymphocyte subset numbers using monoclonal antibodies and measured lymphocyte responsiveness to mitogen during sublethal murine cytomegalovirus (MCMV) infection of 3-wk-old Balb/c mice. MCMV-infected mice had reduced Lyt 1.2 to Lyt 2.2 T-lymphocyte ratios on days 1, 3, 5, and 9 of infection. Alterations in T-lymphocyte subsets were accompanied by diminished lymphocyte response to concanavalin A. Lymphocyte responsiveness and Lyt 1.2 to Lyt 2.2 ratios were maximally reduced on day 5 of MCMV infection and correlated strongly with peak virus recovery from spleen, bone marrow, and peripheral blood leukocytes. These results indicate that acute MCMV infection of mice causes abnormalities in T-lymphocyte subset ratios and responsiveness to mitogen similar to the abnormalities observed in human cytomegalovirus infections. MCMV infection of mice is a useful model to study the mechanism by which cytomegalovirus infections induce altered T-lymphocyte subpopulations. (*Pediatr Res* 19: 583-587, 1985)

Abbreviations

Con A, concanavalin A
 CMV, cytomegalovirus
 MEM, minimum essential media
 MCMV murine cytomegalovirus
 pfu, plaque forming unit
 Lyt 1.2, helper lymphocyte
 Lyt 2.2, suppressor lymphocyte

Observations in humans and experimental animals indicate that infection with CMV adversely affects several parameters of host defense, including B-cell, T-cell, macrophage, and neutrophil functions (1-7). In humans an increased frequency of bacterial or fungal infections occurs in transplant recipients with primary CMV infections (8, 9). Renal transplant patients infected with CMV have reduced helper to suppressor T-cell ratios temporally related to the onset of the viral infection (10, 11) and have been at an increased risk for opportunistic infection when the T-lymphocyte subset was abnormal. Similarly, patients with CMV mononucleosis have altered ratios of T-helper to T-sup-

pressor lymphocytes (12). These alterations in T-lymphocyte subset have been accompanied by diminished lymphocyte responses to mitogen stimulation. Such observations indicate that acute CMV infection alters the function and subset ratio of T-lymphocytes and that altered helper to suppressor ratios among transplant patients may be a marker for an increased rate of secondary infections.

In mice, MCMV infection suppressed the proliferation response of T-lymphocytes to the mitogens phytohemagglutinin and Con A as well as B-lymphocyte proliferation response to bacterial lipopolysaccharide (4, 6, 13). In addition, neutrophil migration and chemotaxis have been decreased during the acute phase of MCMV infection (1). As a result of altered host defense mechanisms, MCMV-infected mice demonstrated enhanced mortality when challenged with a secondary bacterial or fungal agent (2, 3). Previous studies in mice have not investigated T-cell subpopulations during MCMV infection.

To study the effects of CMV infection on T-lymphocyte ratio, we infected 3-wk-old Balb/c mice with the Smith strain of MCMV and measured T-lymphocyte subsets using monoclonal antibodies. Our objectives included: 1) to determine Lyt 1.2 to Lyt 2.2 number and ratio during acute MCMV infection, 2) to measure lymphocyte proliferation response to Con A, 3) to correlate changes in helper to suppressor ratio and lymphocyte function with recovery of infectious virus from blood, spleen, and bone marrow, and 4) to correlate these results with our previous observations regarding the timing of enhanced susceptibility of MCMV-infected mice to secondary infections.

MATERIALS AND METHODS

Animals. Three-week-old female Balb/c mice (Harlan-Sprague-Dawley, Madison, WI) were maintained in a controlled environment (12 h light-dark cycle, food and water *ad libitum*) during these experiments. Infected and control mice were housed separately.

Virus. The Smith strain of MCMV, obtained from Dr. Earl R. Kern, University of Utah, Salt Lake City, UT was used to prepare virus pools. As described previously (2), MCMV was prepared from MCMV-infected mice as a 10% (w/v) homogenate of salivary gland tissue in Eagles' MEM containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (50 g/ml). The MCMV pools contained 1×10^7 pfu of MCMV/ml when titrated on mouse embryo fibroblast cell monolayers. For controls, a normal salivary gland pool was prepared in a similar manner using salivary glands from uninfected 3-wk-old mice.

Animal inoculation. In preliminary experiments, mice were inoculated with serial dilutions of the MCMV pool. A 0 to 20% mortality consistently occurred at an inoculum of 2×10^4 pfu of MCMV/mouse. This inoculum infected all animals as indicated by ruffled fur, diminished activity, and subsequent positive cultures. All deaths occurred on days 7 or 8 of MCMV infection.

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In experiments measuring T-cell subsets and lymphocyte responsiveness, animals received a 0.1-ml inoculum containing 2×10^4 pfu of MCMV intraperitoneal. Control animals received 0.1 ml of a normal salivary gland preparation intraperitoneal.

Leukocyte preparation. On days 0, 1, 3, 5, 9, 16, and 30 after inoculation, groups of eight MCMV-infected and control animals were sacrificed for study. These days were chosen to correlate with previous experiments from this laboratory which studied combined MCMV and bacterial infections (2). Using microcollection pipettes (Unopette, Becton-Dickinson and Co., Rutherford, NJ) and smears of peripheral blood stained with Hemacolor (Harleco, Gibbstown, NJ), total and differential leukocyte counts were determined. Blood for lymphocyte assays was obtained via cardiac puncture and collected in heparinized (10 U/ml) syringes. The mononuclear cell fraction was separated on Ficoll-Hypaque gradients (14) and washed twice with RPMI-1640 with 5% fetal calf serum (GIBCO, Grand Island, NY). The cells were resuspended to a final concentration of 1.0×10^6 cells/ml. Leukocyte viability was $\geq 98\%$ as judged by exclusion of trypan blue.

T-lymphocyte subset determination. Pure monoclonal antibodies directed against Lyt 1.2 and Lyt 2.2 surface antigens of Balb/c mouse lymphocytes (New England Nuclear, Boston, MA) were used to determine the helper (Lyt 1.2) and suppressor (Lyt 2.2) lymphocyte subsets (15, 16). A 0.10-ml aliquot of the mononuclear leukocyte fraction was incubated at 4°C for 30 min with $2.5 \mu\text{l}$ of either Lyt-1.2 or Lyt-2.2 monoclonal antibody. The cells were then washed twice with RPMI-1640. To the cell pellet, $15 \mu\text{l}$ of alkaline-phosphatase conjugated goat antimouse immunoglobulin (Cappel Laboratories, Cochranville, PA) were added, and the cells were allowed to incubate for 30 min. The cells were again washed twice prior to adding 0.25 ml of SIg indicator (Sigma Chemical Co., St. Louis, MO). Following a 30-min incubation at 37°C the cells were resuspended in 1.0 ml RPMI-1640, 0.05 ml 0.12% hydrogen peroxide, and 0.025 ml peroxidase indicator (Sigma Chemical Co.). The cells were centrifuged at $400 \times g$ for 5 min, resuspended in RPMI-1640, and counted using a standard light microscope. Using this technique, monocytes stained black, positive lymphocytes stained red, and negative lymphocytes did not stain. Control samples included cell suspensions incubated with goat antimouse alkaline-phosphatase conjugated immunoglobulin but without monoclonal antibody in order to rule out nonspecific binding of the goat-antimouse IgG to the mouse lymphocytes.

Mitogen-induced proliferation assay. On various days after inoculation with either MCMV or normal salivary gland, mice were sacrificed and peripheral blood from groups of either three infected or three uninfected mice was pooled. Mononuclear leukocytes were isolated on Ficoll-Hypaque gradients, washed, and diluted to a concentration of 1×10^6 viable cells/ml in RPMI-1640 with 10% fetal calf serum. Aliquots (0.1 ml) of the leukocyte suspensions containing 1×10^5 cells/ml were placed into flat-bottom plastic microwells (96 well microtiter plates, Corning, Corning, NY). On days 3 and 5 after MCMV infection, when the mice were leukopenic, groups of six infected or six uninfected mice were pooled to increase cell yield.

Con A, obtained as a lyophilized powder (Sigma Chemical Co.) was dissolved in RPMI 1640 and filter sterilized before use in these experiments. The cells were cultured in triplicate without Con A and with final dilutions of 2.0, 3.0, 4.0 $\mu\text{g}/\text{ml}$ of Con A in a volume of 0.2 ml/well (10). These concentrations of Con A were shown in preliminary experiments to induce maximal stimulation of murine lymphocytes. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 72 h. Eighteen hours before harvesting, $1 \mu\text{Ci}$ of methyl- ^3H -thymidine (specific activity 6.7 Ci/mmol; New England Nuclear) in a volume of 0.02 ml was added to each well. The cell samples were harvested using a multiple sample harvester (Microharvester, Bellco Glass, Vineland, NJ) on fiber glass filters (Whatman, Clifton, NJ), washed with distilled water, air-dried, and immersed in 3.0 ml of Omnifluor toluene scintillation fluid (New England

Nuclear). Samples were then counted in a Beckman LS-100 liquid scintillation counter (Beckman, St. Louis, MO).

Virus assays. On days 1, 3, 5, 9, 16, and 30 of MCMV infection, samples of spleen, blood, and bone marrow were assayed in duplicate for MCMV on confluent monolayers of secondary mouse embryo fibroblast cells in 24-well tissue culture plates (Linbro, Flow Laboratories, McLean, VA) (4). Spleens were prepared as a 10% w/v homogenate in MEM, and peripheral mononuclear cells were obtained by Ficoll-Hypaque separation. For bone marrow cultures, the right femur was removed under sterile conditions and the marrow flushed out with 0.1 ml of MEM. Samples were allowed to adsorb for 1 h and then overlaid with MEM containing 5% fetal calf serum, 100 U/ml penicillin, 50 g/ml streptomycin, and 0.5% agarose (SeaKem, Biomedical Systems, Springfield, NY). Culture data were expressed as log pfu of MCMV per gram of tissue, per 10^6 mononuclear cells, or per milliliter of bone marrow aspirate.

RESULTS

Recovery of MCMV from spleen, blood, and bone marrow. MCMV was recovered from spleen homogenates on days 1 through 16 after inoculation. All MCMV infected mice had MCMV in homogenates of spleen on days 3 and 5 (Table 1). Peak titers of 1.6×10^6 pfu were observed on day 3. Virus was recovered from the mononuclear leukocyte fraction of peripheral blood and from bone marrow aspirates on days 3 through 9. On day 5 virus was recovered from the leukocytes from every MCMV-infected mouse. Titers of MCMV in the leukocyte fraction were similar to previous results studying Swiss-Webster mice (1). After day 9 only one animal had infectious MCMV present in the mononuclear leukocyte fraction.

Characterization of T-lymphocyte subsets. Table 2 summarizes

Table 1. Recovery of MCMV from spleen, peripheral blood mononuclear cells, and bone marrow (mean $\pm$ SD)*

Day after infection	Spleen (log pfu/g)	Mononuclear cells (log pfu/ 1×10^6 cells)	Bone marrow (log pfu/ml)
1	0.75 ± 1.3	0	0
3	6.2 ± 0.53	1.1 ± 0.89	0.83 ± 0.62
5	6.1 ± 0.36	2.4 ± 0.40	1.8 ± 0.12
9	2.1 ± 2.2	0.55 ± 0.95	0.92 ± 0.81
16	2.0 ± 2.6	0.30 ± 0.79	0
30	0	0	0

* Viral assays for infectious MCMV from various tissues document peak titers on day 5 after MCMV infection.

Table 2. Relationship of Lyt 1.2 to Lyt 2.2 T-cell ratio and responsiveness to Con A (mean $\pm$ SD)*

Day	Lyt 1.2:Lyt 2.2		% reduction in Con A response
	Control	MCMV-infected	
0	2.0 ± 1.3		
1	2.3 ± 1.2	$0.9 \pm 0.5^\dagger$	0
3	1.9 ± 0.6	$0.8 \pm 0.2^\dagger$	70
5	2.7 ± 1.4	$0.6 \pm 0.3^\dagger$	93
9	1.5 ± 0.5	$0.7 \pm 0.3^\dagger$	75
16	1.8 ± 0.8	1.2 ± 1.2	62
30	1.2 ± 0.3	1.1 ± 0.7	0

* T-cell subset ratio in uninfected and MCMV-infected mice and relationship to Con A stimulation. The number of circulating lymphocytes reacting with monoclonal antibodies identifying T-cell subset surface antigens was determined during specified days of MCMV infection. The T-cell subset ratio in MCMV-infected mice is significantly depressed during the acute infection. Corresponding with the depressed T-cell subset ratio, a reduction in responsiveness to Con A stimulation occurs.

$^\dagger p < 0.01$ Wilcoxon rank sum test.

Fig. 1. Correlation of T-cell subset ratio to recovery of infectious MCMV from spleen, peripheral blood mononuclear leukocytes, and bone marrow. The depression in the T-cell subset ratio correlated with recovery of infectious virus from bone marrow ($r = -0.95$, $p < 0.02$) and peripheral blood mononuclear leukocytes ($r = -0.82$, $p < 0.05$) on days 3 to 30 of infection.

Table 3. Total lymphocyte count and absolute Lyt 1.2 to Lyt 2.2 T-cell number (mean $\pm$ SD)*

Day	Total lymphocytes		Lyt 1.2 number		Lyt 2.2 number	
	Control	MCMV	Control	MCMV	Control	MCMV
0	1147 $\pm$ 550		458 $\pm$ 235		249 $\pm$ 130	
1	1113 $\pm$ 1056	1500 $\pm$ 1576	403 $\pm$ 361	353 $\pm$ 248	197 $\pm$ 147	514 $\pm$ 414
3	1090 $\pm$ 653	441 $\pm$ 211†	478 $\pm$ 290	136 $\pm$ 72‡	286 $\pm$ 235	164 $\pm$ 68
5	1024 $\pm$ 431	725 $\pm$ 339	351 $\pm$ 165	159 $\pm$ 88†	143 $\pm$ 145	292 $\pm$ 128
9	1694 $\pm$ 1197	3033 $\pm$ 1251	675 $\pm$ 646	740 $\pm$ 630	455 $\pm$ 389	1012 $\pm$ 530†
16	2558 $\pm$ 790	1734 $\pm$ 1543	560 $\pm$ 370	249 $\pm$ 126	381 $\pm$ 246	302 $\pm$ 172
30	1549 $\pm$ 878	3079 $\pm$ 2759	628 $\pm$ 282	1226 $\pm$ 1451	530 $\pm$ 235	1069 $\pm$ 679

* Monoclonal antibody labeled lymphocyte subsets were counted. Decreased helper cell number on days 3 and 5 of infection in MCMV-infected animals accounted for the depressed T-cell subset ratio. However, an increase in suppressor cells on day 9 of infection produced the lowered T-cell subset ratio.

† $p < 0.05$ t test (MCMV infected *versus* control on same day).

‡ $p < 0.02$ t test (MCMV infected *versus* control on same day).

the ratio of Lyt 1.2 to Lyt 2.2 ratios on days 1 through 9 after infection. Beginning the first day after infection with MCMV, the Lyt 1.2 to Lyt 2.2 ratio fell to less than 1.0 with the control values remaining greater than 1.9. On day 5, the ratio reaches its lowest value of 0.6, the same day that infectious virus was uniformly recovered from the spleen homogenates, mononuclear leukocytes, and bone marrow aspirates of all infected animals. Decreases in the Lyt 1.2 to Lyt 2.2 cell ratio appeared to correlate closely with recovery of infectious virus from tissues (Fig. 1). Correlation coefficients between lymphocyte subset ratio and virus recovery were $r = -0.82$ for peripheral blood and $r = -0.95$ for bone marrow.

The lowered T-lymphocyte subset ratios on days 3 and 5 reflected a significant reduction in the absolute number of Lyt 1.2 lymphocytes. On day 9 an increase in the Lyt 2.2 cell subpopulation accounted for the inverted Lyt 1.2 to Lyt 2.2 cell ratio (Table 3). The peripheral white blood cell count showed a significant leukopenia in MCMV-infected mice on day 3 (white blood cells of $863 \pm 371/\text{mm}^3$ in MCMV infected *versus* $2530 \pm 1019/\text{mm}^3$ in controls, $p < 0.01$), followed by a significant leukocytosis on day 9 (white blood cells of $6324 \pm 2775/\text{mm}^3$ in MCMV-infected animals *versus* $2425 \pm 1509/\text{mm}^3$ in controls,

$p < 0.01$, t test). During the first week of infection the MCMV-infected mice also demonstrated a significant lymphopenia, while during the second week of infection, lymphocytosis occurred (Table 3). The latter change corresponded to an increase in the absolute number of suppressor T cells.

Mitogen response of mononuclear leukocytes. Mononuclear leukocyte response to the mitogen Con A was used to measure lymphocyte function and permit correlation with changes in T-cell subset ratio. Data from 9 to 21 mice per day were pooled, and the response of mononuclear cells from MCMV-infected mice was compared to the response of cells from uninfected animals. Mononuclear leukocytes from MCMV-infected mice were significantly less responsive to Con A stimulation on days 3, 5, 9, and 16 after infection than were cells from control animals (Fig. 2).

The reduction in the mononuclear cell response to mitogen correlated with recovery of infectious virus from peripheral blood and with the reduction in the Lyt 1.2 to Lyt 2.2 ratio (Table 2). The greatest reduction in mitogen response occurred on day 5, the same day that the Lyt 1.2 to Lyt 2.2 ratio was maximally reduced. By day 30 of MCMV infection, mitogen responsiveness, as well as the Lyt 1.2 to Lyt 2.2 ratio, had returned to normal.

Fig. 2. Con A response of mononuclear leukocytes from uninfected and MCMV-infected mice at various days of infection. At each day 1×10^5 mononuclear cells were incubated with Con A for 72 h. To evaluate mitogen responsiveness, $1 \mu\text{Ci}$ of tritiated-thymidine ($^3\text{HTdR}$) was added to each microtiter well 18 h before harvest. $^3\text{HTdR}$ incorporation is determined by subtracting the mean cpm of untreated cells from the mean cpm of Con A treated cells of triplicate samples (Δcpm).

DISCUSSION

In the current experiments, acute MCMV infection of 3-wk-old Balb/c mice resulted in a decreased Lyt 1.2 to Lyt 2.2 lymphocyte ratio and diminished responsiveness of peripheral blood lymphocytes to the mitogen Con A. Lyt 1.2 to Lyt 2.2 ratio was reduced 1 day after infection and remained low through day 9. Lymphocyte responsiveness to Con A became abnormal on day 3 of MCMV infection, and this abnormality persisted through day 16. Thus, changes in lymphocyte subset ratio preceded alterations in lymphocyte function. Lyt 1.2 to Lyt 2.2 lymphocyte ratio and lymphocyte responsiveness to mitogens were both maximally reduced on day 5. These abnormalities correlated strongly with recovery of virus from the spleen, peripheral blood, and bone marrow of MCMV-infected animals.

Kelsey *et al.* (4) previously observed maximally suppressed responses of splenic lymphocytes to the mitogens phytohemagglutinin and lipopolysaccharide on days 4 and 5 after MCMV infection with a return to normal by the 15th day. Alloreactivity, measured by the mixed lymphocyte response was suppressed during the first 10 days after infection and also became normal by day 15 (13). Similarly, Osborn *et al.* (7) observed impaired antibody response to an injected antigen on days 3 through 14 after MCMV infection. Thus, immunosuppression by MCMV affects both B-cell and T-cell functions during the first 2 wk of infection.

Suppressed cell-mediated immunity in humans with CMV mononucleosis has been shown by decreased lymphocyte responsiveness to mitogens (5, 17). Carney *et al.* (12) also demonstrated that the helper to suppressor T-cell ratio was decreased during acute CMV mononucleosis and that the abnormal ratio correlated with decreased lymphocyte responsiveness. During convalescence, the T-lymphocyte subset ratio showed a progressive return toward normal and the mitogen-stimulation response also normalized. In additional experiments in humans, Rinaldo *et al.* (18) observed enhanced mitogen stimulation following *in vitro* removal of the suppressor T-cells which had been induced during CMV mononucleosis. This suggested that the depressed immune function correlated with the emergence of increased suppressor T-cells and that a normal response to mitogen could be restored when the excess suppressor cells were removed *in vitro*. Reduced lymphocyte responsiveness and altered helper to suppressor ratio may contribute to the prolonged illness characteristic of some CMV infections.

In the young infant with symptomatic congenital CMV infection, the abnormalities in lymphocyte subpopulations have been less striking. Pass *et al.* (19) observed modestly increased suppressor T-lymphocyte numbers and reduced helper to suppressor ratios in infants less than 1 yr of age. These abnormalities were transient and did not correlate with chronic viral excretion or persistently reduced lymphocyte blastogenic responsiveness to CMV antigens. In contrast, Shannon *et al.* (20) reported persistent mitogen hyporesponsiveness and abnormal T-lymphocyte subpopulations in a young infant with transfusion acquired CMV infection.

Our observations regarding altered helper to suppressor lymphocyte subset ratio during MCMV infection of mice relate most closely to abnormalities which have been noted during CMV infections of human organ and bone marrow transplant recipients (10, 21, 22). Schooley *et al.* (11, 21) observed that abnormal T-lymphocyte subset ratios accompanied CMV infections of renal transplant recipients and identified patients at risk for opportunistic infections with bacterial or fungal pathogens. Inversion of the helper to suppressor T-lymphocyte ratio in this prospective study correlated with the onset of clinically apparent CMV infection, and often preceded the onset of opportunistic infections. In bone marrow transplant patients, Schroff *et al.* (22) observed a strong correlation between declining helper to suppressor T-lymphocyte ratio and CMV infection and Verdonck and DeGast (23) observed similar abnormalities during both latent and primary CMV infections. Although the mechanism by which CMV infection affected the helper to suppressor lymphocyte ratio was not known, Schroff *et al.* (22) speculated that abnormal helper to suppressor ratios in these patients recovering from bone marrow transplantation were attributable to bone marrow suppression induced by CMV infection.

In the murine model, we observed abnormal helper to suppressor lymphocyte subset ratios on days 1, 3, 5, and 9 of MCMV infection. Previous experiments from this laboratory (2) and those of Hamilton *et al.* (3) demonstrated that MCMV-infected mice had enhanced susceptibility to secondary infection with bacterial or fungal agents when mice were challenged with the agents on days 1 through 4 of MCMV infection. Moreover, all deaths during combined MCMV and bacterial infection with either *Escherichia coli* or *Pseudomonas aeruginosa* were observed during days 1 through 10 of MCMV infection. These observations thus suggest that altered Lyt 1.2 to Lyt 2.2 ratios correlate strongly with susceptibility of MCMV-infected mice to secondary infections.

In summary, these experiments demonstrated that acute MCMV infection of mice was associated with inversion of the helper to suppressor T-lymphocyte subset ratio. Inversion of this ratio correlated with reduced lymphocyte responsiveness to Con A and with recovery of MCMV from blood and bone marrow. Although the mechanism for these changes in helper to suppressor ratio is not known, these studies indicate that abnormal helper to suppressor T-lymphocyte ratio was an accurate marker of acute MCMV infection, particularly the viremic phase. Moreover, these animal studies corroborate the observations in humans which suggest that inverted T-lymphocyte subset ratios correlate with an increased risk for opportunistic infections in CMV-infected organ transplant recipients. MCMV infection of mice may be a useful model to elucidate the mechanism by which CMV infections induce altered T-lymphocyte subpopulations.

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Mode of Inhibitory Action of Bilirubin on Protein Kinase C

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ABSTRACT. Ca^{2+} -activated, phospholipid-dependent protein kinase (protein kinase C) is widely distributed in mammalian tissues. Accumulating evidence has revealed that protein kinase C as well as cAMP-dependent protein kinase plays important roles in various cellular functions. The purpose of this study is to examine the effect of bilirubin on protein kinase C and cAMP-dependent protein kinase activity in a cell-free system as a cause of bilirubin toxicity to the central nervous system. Bilirubin inhibited protein kinase C activity in a dose-dependent manner. This effect was markedly diminished by the addition of human serum albumin at a molar ratio of bilirubin to albumin of less than 1.0. Kinetic analysis revealed that bilirubin did not compete with phospholipid, diacylglycerol, or calcium. Bilirubin also inhibited cAMP-dependent protein kinase, but did not compete with cAMP. The inhibitory effect of bilirubin on protein kinase C seems to be irreversible because removal of bilirubin by Sephadex G-25 column chromatography did not restore the protein kinase C activ-

ity. Observations reported herein suggest that bilirubin, especially in its free form, induces an irreversible change to the catalytically active site of protein kinase C. (*Pediatr Res* 19: 587-590, 1985)

Abbreviation

Protein kinase C, calcium-activated, phospholipid-dependent protein kinase

Bilirubin is toxic to the central nervous system, causing encephalopathy, especially in low birth weight premature infants with hyperbilirubinemia and acidosis. The toxic mechanism of bilirubin has been extensively investigated. Several enzymes relating to oxidative phosphorylation and to metabolisms of carbohydrate, amino acid, and lipid are affected by bilirubin (for a review see Reference 1). However, no specific enzyme has been singled out as a target of bilirubin toxicity.

Recent evidence has revealed that protein phosphorylation is a very important pathway in various cellular functions including those of the nervous system (for a review see Reference 2). To

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