Possible activation of murine T lymphocyte through CD98 is independent of interleukin 2/interleukin 2 receptor system

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ABSTRACT

CD98 is a widely expressed cell surface heterodimeric protein of 125 kDa. Its expression is up-regulated during lymphocyte activation induced by mitogen, superantigen, conventional antigen, and a combination of phorbol myristate acetate (PMA) and ionomycin. However, the role of CD98 in the immune system is not so well understood. The role of CD98 in murine T lymphocyte proliferation was investigated, especially in correlation with the interleukin 2 (IL-2)/interleukin 2 receptor (IL-2R) system. Monoclonal antibody (mAb) directed against murine CD98 heavy chain (mCD98HC) suppressed the proliferation of lymphocytes stimulated with concanavalin A (Con A). Anti-mCD98HC mAb did not suppress the expression of IL-2R α . Anti-IL-2R α mAb, which suppressed DNA synthesis, did not inhibit the expression of CD98HC. Murine IL-2 (recombinant), which induced considerable DNA synthesis by lymphocytes stimulated with a sub-optimal dose of Con A, did not induce CD98HC expression in lymphocytes. In addition, anti-mCD98HC mAb did not inhibit the production of IL-2 by lymphocyte stimulated with Con A. Taken together with these findings, it was speculated that the CD98 system is independent of the IL-2/IL-2R system in murine T lymphocyte activation.

Lymphocyte activation process is regulated by many factors such as transferrin (2, 7, 22, 23), insulin (8, 9), interleukin 2 receptor (IL-2R) (CD25) (13) and CD98 (6), and by lymphokines such as interleukin 2 (IL-2) (13, 20, 23, 31). IL-2 is known as a T cell growth factor (13, 20, 31), and IL-2 plays a role in the late G₁ stage (15). IL-2 binds its receptor (IL-2R) (13), and the binding induces proliferation of T lymphocytes. IL-2R is composed of 3 subunits, namely,

IL-2R α (CD25), IL-2R β (CD122) and IL-2R γ (CD132) chains. IL-2R α (55 kDa), known as Tac antigen (28), is specific for IL-2R, while IL-2R β (70-75 kDa) is common for IL-2 and IL-15 mediated signaling, and IL-2R γ (65-70 kDa) is a common subunit of IL-2R, IL-4R, IL-7R, IL-9R and IL-15R. CD98, originally termed 4F2 antigen, is a 125 kDa type II transmembrane protein composed of an 80 kDa glycosylated heavy chain and a 45 kDa non-glycosylated light chain (CD98LC) (6, 24, 25). CD98 is expressed on all activated cells, all cell lines and all known malignant or transformed cells (6, 30). CD98 is a multifunctional protein and its roles are as follows: amino acid transporter (1, 19), integrin regulator (4, 25), fusion regulator (11, 18), in-

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ducer of osteoclast (10), and mouse embryogenesis (27). CD98 is the same molecule as fusion regulatory protein-1 (FRP-1) (17). CD98 also plays an important role in immune cell function and is known as the activated lymphocyte antigen (6, 31). The expression of CD98 on T cells (3) and B cells (30) increases during activation by mitogens such as phytohemagglutinin (PHA) and Con A, superantigens such as *Staphylococcus aureus* enterotoxin A (SEA), conventional antigen such as Tetanus toxoid, and PMA plus ionomycin, or lipopolysaccharide. It was found that CD98 also plays a role in the late G₁ stage of lymphocyte proliferation (3).

Nineteen mAbs directed against mCD98HC were produced (26), and 2 mAbs were found to have inhibitory effects on lymphocytes stimulated with Con A. In the present investigation, the role of CD98HC in murine T lymphocyte activation was analyzed, especially in correlation with the IL-2/IL-2R system.

MATERIALS AND METHODS

Cells. Lymphocytes were isolated from spleens of male C57BL/6 mice of 6–8 weeks old using NH₄Cl lysing buffer. The lymphocytes were cultured at 1×10^6 cells/mL in 96-well round-bottom culture plates in 0.2 mL of RPMI1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% CO₂.

Experiments using mice were carried out according to the guideline for animal experiments in Mie University School of Medicine.

Antibodies and reagents. Monoclonal antibodies against murine CD98HC (26) and an isotype matched control mAb (131A: against NP protein of human parainfluenza type 4A virus) (14) are described elsewhere. MAb against murine IL-2R α (anti-Tac) was purchased from BD Biosciences (Tokyo, Japan) and was dialyzed against sterile PBS to remove NaN₃. FITC-labelled mAbs to murine IL-2R α was from BD Biosciences. Con A and recombinant murine IL-2 were purchased from Calbiochem (La Jolla, California, USA) and Peprotech (Rocky Hill, New Jersey, USA), respectively.

MAb production and purification. MAb against mCD98HC (26–24: IgG2a) and control mAb were produced using VectraCell-Single Use Bioreactor System (BioVectra, Charlottetown, PE, Canada). The hybridoma cells were cultured for about 3 weeks in DMEM supplemented with sodium pyruvate, non-essential amino acids, NCTC109 medium

and 10% FCS.

The supernatant was harvested by centrifugation and filtrated with 0.22 μ m pore filter. The filtrated supernatant was subjected to protein G column chromatography. The eluate was dialyzed against PBS, and the concentration was determined by measuring optical density at 280 and 260 nm.

Labeling of mAb with FITC. Labeling of anti-mCD98HC mAb (11–19) was carried out using the antibody labeling system FITC Labeling Kit K8010 (American Qualex Antibodies, San Clemente, California, USA) according to the manufacturer's protocol.

Proliferation assay. DNA synthesis was measured by cell proliferation ELISA with BrdU Kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's protocol. BrdU (20 μ L/well) labeling solution was added to the lymphocyte culture, and the lymphocytes were incubated for 4 h at 37°C. The plates were centrifuged at 300 g for 15 min, the labeling medium removed by aspiration, and the cells were dried using a hair-dryer. Fix Denat (200 μ L/well) was added to the plates and they were incubated for 30 min at room temperature. Fix Denat was removed by decantation, and anti-BrdU-POD working solution (100 μ L/well) was added. Subsequently, the plates were incubated for 60 min at room temperature. The antibody solution was removed, and the cells were rinsed three times with 200 μ L/well of washing solution. The substrate solution (100 μ L/well) was added to the plates, which were incubated for appropriate times at room temperature. The reaction was stopped by the addition of 1N H₂SO₄ (25 μ L/well). The absorbance at 450 nm was measured with an immunoreader.

FACS analysis. Flow cytometric analysis was performed using FACScan (Becton Dickinson, Tokyo, Japan). For FACS analysis, the lymphocytes were cultured in 3 mL of the medium in a round-bottom glass tube.

Measurement of IL-2. IL-2 was assayed using IL-2 assay Kit (Biosource International, Camarillo, California, USA) according to the manufacturer's method. Fifty μ L of supernatant and 50 μ L of biotinylated anti-IL-2 solution were added to each microplate well, and incubated for 2 h at 37°C. The solution was thoroughly decanted and the wells were washed 4 times with the washing buffer. A hundred μ L of Streptavidine-HRP solution was added to each well

and incubated for 30 min at room temperature. The solution was thoroughly decanted and washed, and 100 μ L of stabilized chromogen was added. Subsequently, the plates were incubated in the dark for appropriate times. A hundred μ L of stop solution was added and the absorbance at 450 nm was read with an immunoreader.

RESULTS

Anti-mCD98HC mAb inhibits the activation of lymphocytes stimulated with Con A

To investigate the function of CD98HC in immune cell responses, we analyzed the effects of anti-mCD98HC mAbs on lymphocyte DNA synthesis induced by Con A. In this experiment, anti-mCD98HC mAb and Con A were added simultaneously. The lymphocytes were cultured with the optimum concentration of Con A (0.7 μ g/well) for DNA synthesis (data not shown) and DNA synthesis was measured at 72 h of stimulation. Out of 19 mAbs against murine CD98HC, 2 mAbs had an inhibitory effect on the proliferation (data not shown). Anti-mCD98HC mAb (26–24) (15 μ g/well) suppressed the mitogenesis by Con A to about 40% (Fig. 1). In contrast, the isotype matched control mAb (IgG2a) (131A: anti-NP protein of human parainfluenza type 4A virus) had no inhibitory effect on the proliferation (Fig. 1).

Fig. 1 Effect of anti-mCD98HC on lymphocyte stimulation. Anti-mCD98HC mAb (26–24) inhibited DNA synthesis by lymphocytes stimulated with Con A to about 40%, but control mAb (131A) had no inhibition on DNA synthesis. DNA synthesis was measured at 72 h of stimulation. The data represents mean $\pm$ SD of 3 experiments.

We used mAb (26–24) (15 μ g/well) in the following experiments.

Relation between CD98HC and IL-2/IL-2R

Firstly, we analyzed the kinetics of IL-2R α expression, CD98HC expression and IL-2 production of Con A-stimulated lymphocytes. The expression of IL-2R α already increased in the early stage, and considerable expression of IL-2R α was observed at 24 h of stimulation (Fig. 2A: red line). CD98HC was up-regulated by Con A-stimulation within several hours, and the maximum expression was also at 48 h post stimulation (Fig. 3A: red line). Resting lymphocytes produced no IL-2. When lymphocytes were stimulated with Con A, the production of IL-2 was observed within several hours and the maximum production of IL-2 (967 pg/mL) was at 48 h post stimulation (Fig. 4).

The mAb against mCD98HC scarcely inhibited the expression of IL-2R α (Fig. 2B: black line).

In the following experiments, we analyzed the effect of anti-IL-2R α mAb on DNA synthesis and on the expression of CD98HC at 72 h and 48 h of Con A-stimulation, respectively. Anti-IL-2R α mAb (15 μ g/well) reduced Con A-induced DNA synthesis to about 60% (data not shown). Anti-IL-2R α mAb did not inhibit the expression of CD98HC (Fig. 3B: red line), and control mAb did not inhibit the expression (Fig. 3B: blue line). From these results, it was indicated that CD98HC and IL-2R α have little functional interaction during proliferation.

Low concentration of Con A (0.1 μ g/well) did not induce notable DNA synthesis by lymphocytes (data not shown). However, addition of recombinant IL-2 (rIL-2) (1000 pg/mL) induced significant DNA synthesis by lymphocytes stimulated with sub-optimal dose of Con A (data not shown). In the following experiment, we analyzed the effect of rIL-2 on the expression of CD98HC at 48 h of stimulation. A sub-optimal dose of Con A induced only a slight expression of CD98HC (Fig. 3C: red line). Recombinant IL-2 (1000 pg/mL) did not enhance the expression of CD98HC on lymphocytes stimulated with a sub-optimal dose of Con A (Fig. 3C: blue line). Recombinant IL-2 itself did not induce CD98HC expression (Fig. 3C: green line). Non-stimulated lymphocytes did not express CD98HC at 48 h, and the figure is almost the same as that of rIL-2 (data not shown).

Fig. 4 shows the effect of anti-mCD98HC mAb on the production of IL-2 at 48 h. Anti-mCD98HC mAb lowered IL-2 production a little (770 pg/mL), and control mAb did also (797 pg/mL). Anti-mIL-

Fig. 2 Anti-mCD98HC mAb did not inhibit the expression of IL-2R α . The representative result out of several experiments is shown. A: Non-stimulated lymphocytes (black line). Con A-stimulated lymphocytes measured at 24 h (red line): considerable expression of IL-2R α was observed. B: Anti-mCD98HC mAb did not inhibit IL-2R α expression (black line). Control mAb had no inhibition (red line).

2R α mAb lowered IL-2 production (610 pg/mL). These results show that anti-mCD98HC mAb had no effect on IL-2 production, indicating that the biochemical process of IL-2 production might not be controlled by the CD98HC system.

DISCUSSION

The inhibitory effect of anti-mCD98HC mAb was described by some investigators using mitogen-, superantigen- and nominal antigen-stimulated peripheral blood mononuclear cells (PBMC) (3, 6, 21, 31). However, the function of CD98HC in immune cell responses has not been so well understood. The present investigation examines the role of CD98HC, especially on the correlation between the CD98 system and IL-2/IL-2R systems. Anti-mCD98HC mAb (26–24) suppressed Con A-stimulated lymphocyte response as much as 40%, and the percentage of in-

Fig. 3 Effects of anti-IL-2R α mAb and recombinant IL-2 on the expression of CD98HC. Anti-IL-2R α mAb did not inhibit CD98HC expression. Recombinant IL-2 (1000 pg/mL) did not enhance CD98HC expression in lymphocytes stimulated with a sub-optimal dose of Con A. The representative result out of several experiments is shown. A: Non-stimulated lymphocytes (black line) measured just after preparation. Con A-stimulated lymphocytes (red line) measured at 48 h: considerable expression of CD98HC was observed. B: Non-stimulated lymphocytes (black line). Anti-IL-2R α mAb did not inhibit expression of CD98HC (red line). Control mAb had no inhibition (blue line). C: Non-stimulated lymphocytes (black line). Sub-optimal dose of Con A-stimulated lymphocytes (red line) at 48 h: little expression of CD98HC was observed. Sub-optimal dose of Con A- and rIL-2-stimulated lymphocytes (blue line): recombinant IL-2 did not enhance the expression of CD98HC. Lymphocytes stimulated with only rIL-2 (green line): little expression of CD98HC was observed.

Fig. 4 Anti-mCD98HC mAb had no effect on IL-2 production. Anti-mCD98HC mAb lowered IL-2 production a little, but control mAb also lowered IL-2 production to the same concentration. Anti-mIL-2R α mAb lowered IL-2 production. These results indicated that anti-mCD98HC did not inhibit IL-2 production. IL-2 production was measured at 48 h of stimulation. The data represent mean $\pm$ SD of 3 experiments.

hibition was about the same as that found by other investigators. Haynes *et al.* (6) reported that 4F2 antibody inhibited mitogen-induced tritium thymidine incorporation of PBMC by 50%. Diaz *et al.* (3) reported that anti-CD98HC mAb (4F2) inhibited the superantigen response by as much as 80%. The inhibition levels of rIL-2-mediated mitogenic response by anti-CD98HC mAb were varied from minimal to as much as 50% inhibition (3). Both mAb UM7F8 (anti-CD98HC) and 4F2 completely blocked the response to Tetanus toxoid (3). Exogenous conventional antigen needs to be processed by antigen presenting cells (APC) such as monocytes, while superantigen and mitogen do not require processing for presentation to helper T cells, indicating that the degree of inhibition depends on the kind of stimulants.

In addition, 4F2 did not inhibit PBMC response to PMA plus ionomycin or rIL-2 (3), though CD98HC was expressed with stimulation of PMA plus ionomycin (29), indicating that the stimulation process without APC may not require CD98, and the CD98 system is involved in only a co-stimulatory process. Stonehouse *et al.* (21) showed that mAb to CD98 and CD147 showed an inhibitory effect when prepulsed on to the U937 cells, suggesting that the inhibitory effect was mediated by these molecules on the APC. Adhesion between U937 and T cells may be a prerequisite for proliferation. Diaz *et al.* (3) also showed the role of monocytes in the inhibition of anti-CD98HC mAb: they showed that treatment of PBMC with L-leucine methyl ester, which depletes phagocytic cells, abrogated the inhibitory effect of anti-CD98HC mAb on T cell proliferation. These results indicated that anti-CD98HC mAb acted on APC, and CD98HC played a role in presenting antigen on MHC class II molecules to T cells. CD98HC did not regulate the initial activation events involving the interaction between the APC and T cells. This is supported by the fact that anti-CD98HC mAb does not affect Ca^{2+} fluxes by SEA. In addition, the fact that 4F2 strongly inhibited T cell response even when added 18 h or more post stimulation, suggested that CD98 was not involved in early signaling, and acted on T cells in the late G_1 phase. Anti-CD98HC mAb may block the binding of a certain molecule to CD98HC in monocytes, but not to CD98HC in T cells. One of the candidate molecules was galectin 3 (5). Galectin 3 is secreted from monocytes but not from T cells. It was found that galectin 3 binds to CD98 and induces Ca^{2+} fluxes, and also activates monocytes and induces IL-1 secretion (16). Anti-CD98HC mAb may block monocyte auto-activation by galectin 3.

In this study, anti-mCD98HC mAb neither inhibited IL-2 production nor IL-2R α expression induced by Con A. However, there is a disagreement concerning the effect of anti-CD98HC mAb on IL-2R α expression. Diaz *et al.* previously reported that anti-CD98HC mAb inhibited IL-2R α expression induced by superantigen, but did not reduce expression of the IL-2 (3). A possible explanation for the discrepancy between our result and the result of the other investigator (3) is as follows: superantigen makes a bridge between the MHC class II molecule and the T cell receptor, but it does not bind to the cell membrane of the two cells, and this process may be important for the abrogation of IL-2R α expression of T cells. On the other hand, mitogen binds some part of the cell membrane of APC and T cells, and

T cells may express IL-2R α independently of CD98HC of APC. The experimental result that PHA-induced blast cells cultured with rIL-2 were partially inhibited by anti-IL-2R α mAb (3) is similar to our result that anti-IL-2R α mAb could suppress Con A-induced proliferation by as much as 40%. Anti-CD98HC mAb did not block the proliferation of pre-activated lymphocytes (3), due to production of IL-2 and expression of IL-2R α in pre-activated lymphocytes. Pre-activated lymphocytes proliferate via the IL-2/IL-2R system, and the proliferation does not depend on the CD98HC system. From these results, it is inferred that the CD98 and IL-2/IL-2R systems are independent and that the CD98HC system is an additional secondary co-stimulating system in immune response.

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