Interleukin-2 Enhancement of Cytotoxicity by Humanized Monoclonal Antibody M195 (Anti-CD33) in Myelogenous Leukemia

Philip C. Caron, Lawrence T. Lai, and David A. Scheinberg

Department of Medicine, Clinical Immunology and Leukemia Services, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

Humanized M195 (HuM195) is a genetically engineered, human IgG1 version of the parent M195, a mouse immunoglobulin G2a, anti-CD33 monoclonal antibody which reacts with early myeloid progenitor cells and myelogenous leukemia cells. In Phase I studies in patients with relapsed and refractory myelogenous leukemia, HuM195 safely targeted to sites of disease and was nonimmunogenic. HuM195 shows only modest capability of antibody-dependent cellular cytotoxicity (ADCC) against target HL60 cells and minimal cytolytic activity mediated by human complement. Therefore, efforts were made to enhance ADCC using cytokines. γ-Interferon, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor did not promote neutrophil-mediated ADCC with HuM195. However, interleukin-2 (IL-2) showed a range of 2-6-fold increases in ADCC against fresh myelogenous leukemia cells and HL60 cells over that seen with HuM195 or low-dose IL-2 alone. ADCC potency was not improved further by the use of homodimeric HuM195. Flow cytometry and Fc receptor-blocking experiments showed that CD16+ cells were essential for IL-2-enhanced ADCC. As compared to HL60 cells, a multidrug-resistant line of HL60 cells was at least as susceptible to killing by IL-2 or HuM195 or in combination, suggesting that the mechanism of killing may be active against cells surviving and resistant to chemotherapy. Since these in vitro levels of IL-2 and HuM195 can be safely achieved in patients, the enhancement of HuM195 ADCC with low-dose IL-2 is a possible strategy that may be used in vivo to eliminate minimal disease in future trials of patients with myeloid leukemias.

INTRODUCTION

Chimeric and humanized mAbs3 show new immune effector functions not seen in their murine counterparts (1-5). CDR-grafted HuM195 is a human IgG1 that was derived from M195 (6), a mouse IgG2a, anti-CD33 mAb reactive with early myeloid and myelogenous leukemia cells, but not early hematopoietic progenitor cells (7, 8). Unlike its murine parent mAb, HuM195 shows a higher affinity for its antigen and the new capability of modest ADCC against HL60 leukemia cells using human PBMCs as effectors (9).

Although therapy with radiolabeled M195 has shown specific targeting and effective cytoreduction in patients with myelogenous leukemia (10-12), the need for radioactivity and its inherent toxicities complicates the therapeutic potential of HuM195. The in vitro cytotoxicity of unlabeled HuM195 has led to its investigation in human trials. Specific targeting of sites of disease was seen in 13 patients with refractory or relapsed myelogenous leukemia that were treated in a Phase I trial using trace-labeled HuM195 (13). HuM195 did not have potent antileukemic activity alone in this setting. Toxicity was mild and reversible and, most important, no immunogenicity was found up to 4 months after repeated HuM195 administrations.

IL-2 is well known to have a major role in the regulation of cellular immune responses and antitumor activity (14). IL-2 can augment ADCC using murine or chimeric mAbs (15, 16) as well as a bispecific mAb (17). However, other than with anti-Tac-H, a humanized version of murine anti-Tac against the low affinity receptor for IL-2 (3), little work has been done with IL-2 to further increase cell killing by humanized mAbs. Immune effector function with HuM195 has been modest (9). Previously, we have been able to significantly improve ADCC potency by the construction of a dimeric HuM195 (18), but the feasibility of utilizing dimeric HuM195 in clinical trials has yet to be determined.

In addition to IL-2, GM-CSF enhanced ADCC against melanoma cells (19, 20), and showed activity against colorectal cancer possibly due to augmentation of ADCC (21). Both G-CSF and GM-CSF enhanced the cytotoxic function of neutrophils against human immunodeficiency virus-infected cells (22). IFN-γ has been demonstrated to increase ADCC of fresh, chronic lymphocytic leukemic cells (23). Others have shown
that additional cytokines in combination can augment IL-2-induced ADCC (24).

Since regimens of low-dose IL-2 have recently been designed that can be safely given to humans in vivo (25–27), we chose to study whether HuM195 in conjunction with IL-2 would enhance leukemia cell killing in vitro. Other cytokines such as IFN-γ, GM-CSF, and G-CSF are also examined. In this article, the enhancing effects of IL-2 on HuM195-mediated ADCC on HL60 cells, fresh leukemia cells, and a MDR cell line developed from HL60 cells (28) are described. The specificity and mechanism of action of IL-2-enhanced ADCC were studied by blocking Fe receptor binding and by using flow cytometry to determine which cell types were involved in cell killing. In addition, a dimeric HuM195, previously shown to be significantly more potent than the monomer at ADCC (18), was studied in combination with IL-2.

MATERIALS AND METHODS

Cells. Hematopoietic cell lines, including HL60, K562 (NK cell-sensitive), and Daudi (NK-resistant) cells, maintained at Sloan-Kettering Institute, were grown in RPMI 1640–5% newborn calf serum-10% serum-plus (Hazelton Biologics, Inc., Lenexa, KS). Heparinized peripheral blood samples were obtained from healthy volunteers and patients on the Leukemia Service at Memorial Hospital with Institutional Review Board-approved protocols. PBMCs and leukemic blasts from patients with newly diagnosed, untreated myelogenous leukemia were separated on Ficoll-Paque (Pharmacia, Piscataway, NJ), washed twice with RPMI 1640, and resuspended in RPMI 1640–5% newborn calf serum-10% serum-plus. Neutrophils were separated using neutrophil isolation medium (Cardinal Assoc., Inc., Santa Fe, NM), washed twice in phosphate-buffered saline, and resuspended in RPMI 1640–10% fetal calf serum-10% serum-plus (Hazelton Biologics, Inc.). Purity of neutrophils was determined by morphology and flow cytometry. A MDR HL60 cell line HL60/RV + was kindly provided by Dr. Melvin Center (Kansas State University, Manhattan, KS; Ref. 28).

mAb. mAb M195 (anti-CD33) originated in BALB/c mice immunized with leukemia cells from a patient with AML, and was produced from hybridomas and purified as described previously (7, 8). CDR-grafted HuM195, retaining only the CDRs and other sterically important amino acids from the mouse IgG1, was constructed by computer modeling and genetic engineering as described (6). Purity was determined on sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie brilliant blue. HdM195 was constructed as described (18).

Antibody-dependent Cellular Cytotoxicity. Chromium release assays were conducted as described previously (9) using PBMCs or neutrophils from human volunteers as effector cells and HL60, HL60/RV +, and fresh leukemia cells as positive targets. HuM195 concentrations of 0.2, 1, 5, or 10 μg/ml were used at E:T ratios of 12.5–100:1. Effector cell only- and mAb only-treated target cells were used as negative controls. Controls using Fd79, an unrelated, isotype-matched humanized IgG1 (4), did not show any activity (data not shown). Samples were done in quadruplicate, and SDs were <10% of the mean value. Specific lysis = A - C/B - C, where A = cpm release in the presence of medium alone, B = total cpm released by detergent lysed cells, and C = cpm released in the presence of medium alone.

IL-2 Enhancement of ADCC. To investigate the effect of IL-2 (Boehringer Mannheim, Indianapolis, IN) on HuM195- or HdM195-mediated ADCC, effector cells were incubated at 37°C at final concentrations of IL-2 ranging from 2 to 200 units/ml. In most experiments, effector cells were preincubated for 18 h, but in some cases for up to 4 days prior to mAb administration as noted. Target cells included fresh leukemia cells as well as HL60 and MDR HL60/RV + cells. Killing by NK cells, as defined as the amount of killing seen with effectors alone, was also measured against the NK-sensitive K562 cell line. LAK activity, as defined by the amount of killing seen with IL-2 and effectors alone, was measured against the NK-resistant Daudi cell line.

Fc Receptor-blocking Experiments. Chromium release assays with IL-2-primed effector cells were tested with HuM195 with or without an IgM anti-CD16 mAb (anti-FcγRII or Leu-11b) (Becton Dickinson, San Jose, CA) at the same concentration of 1 μg/ml as HuM195. A control IgM, anti-CD15 M31 (prepared in the laboratory), was used as a control. The percentage of inhibition of ADCC that could be attributed to HuM195-specific lysis was measured.

Flow Cytometry and Radioimmunoassay. MDR HL60/RV + cells were analyzed for cell surface CD33 antigen sites by radioimmunoassay as described previously (13) and by using an EPICS Profile II flow cytometer (Coulter Co., Hialeah, FL) according to previous methods (8). Double marker analyses of human PBMCs were performed using fluorescein isothiocyanate- and phycoerythrin-conjugated mAbs, including Leu-11a (CD16) (FcyRII) (Becton Dickinson), NK1I-1 (CD56), B4 (CD19), IL2R1 (CD25), T3 (CD3), T8 (CD8), and isotype-matched control mAbs (Coulter Co.). On Day 1, fresh human PBMCs (suspected in RPMI 1640 with 20% bovine serum albumin) that were cultured overnight with or without IL-2 in Teflon vials (Savillex Corp., Minnetonka, MN) were double stained and analyzed.

Cytokines G-CSF, GM-CSF, and γ-IFN and HuM195-Neutrophil-mediated ADCC. In addition to IL-2, the cytokines IFN-γ (Genetech Inc., Palo Alto, CA), GM-CSF (Immunoex Corp., Seattle, WA), and G-CSF (Amgen Inc., Thousand Oaks, CA) were tested with HuM195 using neutrophils incubated with cytokines either overnight or 2 h before starting the ADCC assays. Viability was tested. Cytokine concentrations were either 100 or 200 units/ml, and in the case of GM-CSF, a range of 20–1000 units/ml was used, with HuM195 varying from 1 to 10 μg/ml.

Statistical Methods. Standard deviations of means are presented as bars in Figs. 1–5. The data were analyzed by a paired r test.

RESULTS

IL-2 Enhancement of HuM195-mediated ADCC. Killing with HuM195 alone was usually about 20% as reported previously (9). When PBMCs (i.e., effectors) were incubated for 18 h with IL-2 at 50 or 100 units/ml at 37°C, ADCC with HuM195 at 1 or 5 μg/ml was augmented in the range of 2–6-fold using HL60 cells as targets at E:T ratios of 25, 50, and
100:1 (n = 11 experiments). Approximately 60–80%, and in some cases >90%, of HL60 cells were killed with IL-2 and HuM195. Longer time periods of incubation (4 days) with IL-2 under the same conditions showed similar results with HL60 cells (results not shown).

In most cases, LAK activity represented about one-third of the killing seen for HuM195 and IL-2 together, with great variability ranging from 10 to 70%, depending on the donor cells. Higher LAK values were seen with increasing E:T ratios. NK activity was less than 5% of the total killing. NK-sensitive K562 cells were killed effectively by effectors alone (50–70%) or in combination with IL-2 (70–80%). NK-resistant Daudi cells were killed by effectors in combination with IL-2 (70–90%), but not by effectors alone.

A representative experiment with HL60 cells is shown in Fig. 1A. The increase in ADCC with IL-2 (50 units/ml) and HuM195 (5 μg/ml) in combination was additive at the lower E:T ratios (25 and 50:1), and less than additive at the highest E:T ratio (100:1) over that seen with HuM195 or IL-2 alone. The increase in ADCC with IL-2 and HuM195 was significantly greater (P < 0.01) compared to either alone at each E:T ratio.

When fresh leukemia cells from patients with untreated AML were used as target cells, killing with HuM195 was also increased significantly (P < 0.01) by the addition of IL-2 (Fig. 1B). LAK activity remained less than 10%. No killing was seen with effectors alone. While killing with HuM195 alone was 10–20%, HuM195-mediated ADCC in the presence of IL-2 increased this value to 30–60%, representing a 2–4-fold enhancement. At each E:T ratio, the amount of IL-2-enhanced ADCC was greater than the sum of ADCC observed with HuM195 or IL-2 alone.

ADCC as a Function of HuM195 and IL-2 Concentrations. Dose-dependent effects of antibody concentrations were examined. No difference in ADCC was seen at 1, 5, or 10 μg/ml of HuM195 with or without IL-2, while ADCC was submaximal at 0.2 μg/ml (results not shown). ADCC with HuM195 at 1 μg/ml was measured at IL-2 concentrations ranging from 5 to 200 units/ml. At each E:T level, a plateau of killing occurred beginning at 20–50 units/ml (Fig. 2). This result was confirmed in 4 other experiments, where killing was always found to be equivalent for IL-2 levels of 50 units/ml or higher. Killing with IL-2 alone was usually <10% at IL-2 levels of 25 units/ml or below, and about 20–35% for IL-2 levels above that amount.

Flow Cytometry of IL-2-enhanced Effector Cells. PBMCs from donor samples were separated using flow cytometry by gating small, agranular cells (Population 1) and larger, more granular cells (Population 2) based on forward and side scatter, before and after stimulation with IL-2 at 50 or 100 units/ml (Fig. 3A). Effector cells were incubated in Teflon-coated vials so all cells involved in ADCC could easily be measured (n = 4 experiments). No significant change can be seen in CD16,CD56 dual positivity in Population 1 after an 18-h incubation with IL-2. After an 18-h incubation at 37°C in the absence of IL-2, only 2% of Population 2 consisted of CD16+,CD56+ cells. However, cells coexpressing CD16 and CD56 consistently represented approximately 15% of the total PBMC after an 18-h incubation with IL-2 (Fig. 3, B and C). Although CD3+ cells showed a moderate increase (30%), no changes in CD8, CD19, or CD25 positivity were seen with IL-2 in either population.

Inhibition of ADCC by Anti-FcγRIII. The mechanism of ADCC involves specific Fc receptor attachment to target cells (29). Thus, blocking Fc receptor sites with an anti-CD16 IgM mAb (anti-FcγRIII) would inhibit specific ADCC not attributed to IL-2 killing alone. Anti-CD16, a murine IgM, inhibited IL-2-enhanced HuM195-mediated ADCC by 70–90% at the same concentration of 1 μg/ml used for HuM195 (P < 0.02; Fig. 4).
No inhibition was seen in the presence of a control IgM (anti-CD15, M31) in combination with HuM195. Inhibition was seen at each E:T ratio. LAK activity was not affected by the presence of anti-CD16. Duplicate experiments confirmed these results.

**ADCC with HdM195.** Previously, a homodimeric version of HuM195 (HdM195) was shown to be significantly more potent (100-fold) at complement-mediated cytotoxicity and ADCC of HL60 cells (18). In a series of 6 experiments with overnight incubation of effector cells with IL-2, killing of HL60 cells with HdM195 was equivalent or slightly higher than with HuM195 (results not shown). Killing was typically in the range of 60–80% with either HuM195 or HdM195 and IL-2 as low as 20 units/ml at E:T ratios above 50:1. Similar levels of killing with HuM195 and HdM195 were seen at each E:T ratio and mAb concentrations of 0.2, 1, and 5 μg/ml.

**Immunological Killing of MDR Cell Line.** The MDR HL60/RV" cell line was tested to see whether cells were more resistant to killing by IL-2, HuM195, or a combination of IL-2 and HuM195. IL-2-enhanced HuM195-mediated ADCC in both cell lines was above that seen with HuM195 or IL-2 alone. In a total of 6 separate experiments using different donors, HL60/RV" cells were at least, or in some cases, more susceptible to killing with HuM195 (1 or 5 μg/ml) or IL-2 (12.5–100 units/ml), alone or in combination, compared to HL60 cells.

Fig. 5 shows a representative experiment with HL60/RV" cells using HuM195 (1 μg/ml) and IL-2 (50 units/ml) at an E:T ratio of 50:1. IL-2 enhanced killing of HL60/RV" cells compared to either IL-2 or HuM195 alone (P < 0.01). In this case, killing of HL60/RV" compared to HL60 cells was significantly greater (P < 0.03) with either IL-2 or HuM195 alone, or in combination. When HdM195 was tested, there was no additional enhancement of IL-2-stimulated ADCC of the MDR cell line compared to HuM195, with 50–80% of the cells eliminated at a mAb concentration of 1 μg/ml and IL-2 at 100 units/ml. Flow cytometric analysis and radioimmunoassay showed that HL60/RV" cells had 50% more cell surface CD33 sites than HL60 cells (results not shown).

**IFN-γ, GM-CSF, and G-CSF Effects on Neutrophil-mediated ADCC.** ADCC of HL60 cells with HuM195 and neutrophils (determined to be 98% pure by morphology and >92% pure by flow cytometry using Leu-11a-fluorescein isothiocyanate) was not consistent and always <15%. Neutrophils that were incubated overnight were >95% viable using trypan blue exclusion. Overnight incubation of neutrophils with IFN-γ at 100 or 200 units/ml did not show consistent activity with HuM195 at 1 or 10 μg/ml and E:T ratios of 25, 50, and 100:1 (n = 7 experiments). Neither GM-CSF (n = 12) nor G-CSF (n = 5) at similar concentrations, when incubated overnight or 2 h prior to starting the assay, were consistently effective at promoting ADCC with HuM195 at 1–10 μg/ml. A more detailed analysis with GM-CSF at concentrations ranging from 20 to 1000 units/ml also failed to show any activity.

**DISCUSSION**

The purpose of this article was to develop a strategy for effective killing of leukemia cells in vivo using a humanized mAb. A number of therapeutic clinical trials of mouse monoclonal antibodies have been performed in hematological malignancies, but the lack of potency and efficiency and the immunogenicity of these mouse IgGs has prevented major success (30). HuM195, an anti-CD33, IgG1 mAb with a higher avidity and new capability of ADCC compared to the original murine version (9), has been shown to be safe and nonimmunogenic in multiple doses in a Phase I trial for patients with relapsed and refractory myelogenous leukemia (13). However, the amount of ADCC in vitro with HuM195 has been modest. We have tested a number of cytokines with HuM195 and a dimeric HuM195 to try to enhance ADCC to a level that may be clinically relevant. In our studies, compared to HuM195 or IL-2 alone, no other cytokine except IL-2, including IFN-γ, GM-CSF, and G-CSF, demonstrated significant antileukemic effects or enhancement of ADCC.

IL-2 alone was shown to be active in some studies against a low burden of disease in AML (31) or when used to improve graft versus leukemia effect in the postallogeneic bone marrow transplant setting (32). However, the killing that is seen with HuM195 and IL-2 shown here is specific and effective at levels of IL-2 as low as 20 units/ml. While early trials with IL-2 involved severe toxicity related to high doses, clinical trials demonstrate that low-dose continuous infusion IL-2 can be used with reduced toxicity compared to bolus i.v. dosing (27). The toxicity of IL-2 in vivo depended on the dose, schedule, and route of administration when studied in solid tumor patients (33). Other methods of IL-2 administration, such as s.c. IL-2, were well tolerated (34).

Low IL-2 doses resulted in the accumulation of a population of lymphocytes consisting primarily of NK cells characterized as CD3−,CD56+, with the majority coexpressing CD16+ after 8 weeks of therapy (26). In our assay, the accumulation of cells coexpressing CD16 and CD56 in the large granular cell population probably represents activated lymphocytes. This
Flow cytometry of IL-2-stimulated effector cells. PBMCs were incubated with or without 50 units/ml of IL-2 for 18 h. A, location of Population 1 (small, agranular cells) and Population 2 (larger, granular cells) on a forward versus log side scatter dot plot. B and C, logarithmically increasing fluorescence intensity of fluorescein plotted in 1024 channels on the X-axis and phycoerythrin plotted on the Y-axis. B, relative absence (2%) in Box 2 of a dual staining population of CD16+,CD56+ cells incubated at 37°C for 18 h in the absence of IL-2. C, accumulation of cells coexpressing CD16 and CD56, representing approximately 15% of the total PBMCs after 18 h of IL-2 stimulation.

In our hands, ADCC with HuM195 was not mediated by neutrophils, even after stimulation with GM-CSF, G-CSF, or IFN-γ. Although G-CSF and IFN-γ induce FcRI on neutrophils (29), it is possible that the Fc region of HuM195 does not effectively bind to neutrophil Fc receptors, explaining the lack of ADCC. The enhancement of ADCC by GM-CSF with monoclonal antibody 3F8 mediated by neutrophils against neuroblastoma cell lines correlated with an increased expression of CD11/CD18 adhesion molecules (36).

HuM195 at 1 μg/ml was as effective as higher doses at optimal killing with IL-2 levels above 20 units/ml. Since the subpopulation of NK cells possessing the FcγRIII receptor was most likely responsible for the ADCC with HuM195, as evidenced by the amount of inhibition seen in blocking experiments with anti-CD16. Others have shown that blocking CD16 eliminated ADCC (15, 16), as also demonstrated by the lack of augmentation of ADCC by IL-2 with F(ab')2, anti-idiotypic fragments (35). Although CD3+ cells increased after IL-2, we could not confirm these to be the same as the CD16+,CD56+ cells without triple staining. Although low-dose IL-2 did not cause an accumulation of CD3+ cells, CD3+,CD25+ cells are involved in T cell activation (33).
Fig. 4 Effect of anti-CD16 on IL-2-stimulated ADCC with HuM195. ADCC assays against HL60 cells using human PBMCs as effector cells at E:T ratios of 25, 50, and 100:1 were run with HuM195 at 1 μg/ml alone (■), HuM195 and IL-2 at 50 units/ml (□), or both in the presence of a control anti-CD15 IgM (△) or an anti-CD16 IgM at 1 μg/ml (▲). This was shown in two separate experiments with different effector cells.

Fig. 5 ADCC of multidrug-resistant leukemia cells using IL-2 and HuM195. The MDR HL60/RV + leukemia cells (■) were compared to HL60 cells (▲) for their susceptibility to killing by ADCC with HuM195 at 1 μg/ml. Human PBMCs were incubated with IL-2 at 50 units/ml for 18 h and 5-h chromium release assays were performed at an E:T ratio of 50:1. The results shown here are from a single experiment (see text).

The avidity of HuM195 is high and the number of cell surface antigen sites is low, it is not surprising that a concentration-dependent curve was not seen. It was previously shown that higher amounts of mAb did not necessarily mean a greater degree of ADCC, presumably due to an increased level of antibody-antigen interaction and loss (i.e., modulation) from the cell surface (9). In the Phase I trial of trace-labeled HuM195, the intermediate dose of 3 mg/m² was found to be the optimal biological dose based on imaging studies and flow cytometry, with higher doses showing blood pooling and saturation of antigen sites (13). Peak HuM195 levels at this dose were 0.5 μg/ml, and in the range that showed effective ADCC in these in vitro studies.

The HdM195 (18) did not show a significant improvement in ADCC over its monomeric counterpart when tested in conjunction with IL-2. However, the amount of ADCC seen with IL-2 and either HuM195 or HdM195 was still greater than the improved potency of HdM195 over that of HuM195 reported previously (9). Hence, the enhanced ability of IL-2-stimulated effector cells to kill target cells more than compensated for the proximity of Fc receptors thought to explain the greater ability of HdM195 alone to perform ADCC.

One of the major obstacles to treatment of AML is the high rate of relapse, in part due to the emergence of MDR leukemia cells (37). The development of a MDR HL60/RV + cell line (28) that was used in this article has allowed us to study its susceptibility to other methods of killing besides modifying the MDR phenotype. The ability of HuM195 and IL-2 to kill a MDR cell line at least as well as the chemotherapy-responsive HL60 cells has far reaching consequences. The explanation for the possibly increased susceptibility of the MDR + cell line to IL-2 killing alone is not readily evident. The increased CD33 expression observed may allow for improved killing with HuM195 alone or in combination with IL-2 due to improved Fc interactions. We have previously shown in CD33-enhanced fibroblasts that the degree of both ADCC and complement-mediated cytotoxicity correlated with antigen density (9).

The augmented ADCC seen after an 18-h IL-2-incubation would be similar to that seen in i.v. boluses. This is most likely due to the increased perforin and granzyme activity induced by IL-2 (38). Recently, it was shown that granzymes secreted by cytotoxic lymphocytes trigger an internal disintegration pathway leading to cell lysis and DNA breakdown (39).

Based on these promising in vitro results, a strategy for the eradication of leukemia cells in vivo can be developed. (a) The number of CD16 +,CD56 + cells necessary for ADCC will be expanded by a low-dose continuous IL-2 infusion or by s.c. administration. (b) This will be followed by intermittent high-dose IL-2 boluses prior to repeated antibody dosing to increase cytolytic activity in an attempt to reproduce the in vitro results. Cells from patients already exposed to a number of cytotoxic agents may still be expected to be susceptible to HuM195 and IL-2. The advantage of HuM195 over murine and chimeric mAbs is that repeated courses of therapy may be given due to the lack of immunogenicity.
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