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Award Number: DAMD17-96-1-6107

TITLE: Antibody-IL2 Fusion Protein Delivery by Gene Transfer

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (01 Oct 98 - 30 Sep 99)

4. TITLE AND SUBTITLE
Antibody-IL2 Fusion Protein Delivery by Gene Transfer

5. FUNDING NUMBERS
DAMD17-96-1-6107

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Over the past year our team has been actively investigating the immunotherapeutic potential of the antibody-cytokine fusion proteins. These molecules contain the antibody portion recognizing the tumor associated antigens, and is covalently linked to a potent immune stimulator. HuKS-IL2 fusion protein which is highly reactive with breast cancer cells became available to us for *in vitro* and in animal *in vivo* studies. We are still actively pursuing experiments to elucidate the mechanisms of targeting tumor cells for destruction and mechanisms of stimulation immune effector cells by this molecule, to ultimately translate these findings to clinical application. At this time similar fusion protein (hu14.18-IL2) became available for clinical testing and we decided to refocus our efforts in this direction. At the UW-CCC we are currently performing an initial Phase I clinical trial involving an administration of this novel immunocytokine to the patients with GD-2 positive tumors, delivered as a single agent therapy. We are collecting serum specimens as well as cells from these patients. Studies are underway to assess the effects of this treatment, its safety and future applications. Findings from these studies will provide a baseline for clinical testing of other immunocytokines targeting human cancers.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	19
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Jacki Gan — 10/29/99

PI - Signature — Date

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List of abbreviations :

ADCC	Antibody Dependent Cellular Cytotoxicity
ELISA	Enzyme-Linked Immunosorbent Assay
EpCAM	Epithelial Cell Adhesion Molecule
FP	Fusion Protein
GD-2	Disialoganglioside
Hu14.18-IL2	Humanized 14.18 antibody/interleukin-2 fusion protein
IC	Immunocytokine
IFN-γ	Interferon gamma
IL-2	Interleukin 2
IL-2R	Interleukin-2 receptor
LAN-5	Human neuroblastoma cell line
M-21	Human melanoma cell line
mAb	Monoclonal Antibody
NK	Natural Killer Cell
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
sIL-2Rα	Soluble Interleukin-2 receptor Alfa Subunit
UWCCC	University of Wisconsin Comprehensive Cancer Center

INTRODUCTION :

Over the past year we have been actively investigating the immunotherapeutic potential of antibody - cytokine fusion proteins. In these molecules the antibody portion recognizes the tumor associated antigen, and is covalently linked to potent immune stimulator. One of these fusion proteins, huKS-IL2, which is highly reactive with breast cancer cells, became available to us in sufficient quantities to pursue the pre-clinical *in vitro* and *in vivo* studies. Most of our findings in this experimental system were described in previous progress report. We are still actively pursuing these experiments and are interested in elucidating the mechanisms of targeting tumor cells for destruction directly or by attracting and stimulating resident immune effector cells. Our ultimate goal is to determine how this molecule is likely to be effective in treating cancer patients in clinical settings and which treatment regimen will be most effective. Unfortunately this molecule is not yet available for clinical testing, so together with expanding the *in vitro* experiments with KS-IL2 fusion protein, we refocus our attention into different direction.

Hu14.18-IL2 fusion protein, from the same family of molecules but of different specificity became available for clinical studies. At the UW-CCC we are currently performing an initial Phase I clinical trial of this novel immunocytokine, delivered as a single agent therapy to the patients with GD2 positive tumors. We are collecting serum specimens as well as cells from these patients to study the effects of this treatment, its safety and future applications. Our goal is to document that this fusion protein is clinically safe and well tolerated at doses that induce striking immunologic activation. Some of preliminary findings from our laboratory testing of these specimens collected from initial patients are described in this report.

BODY :

The following is a summary of progress we have made over the past year with KS-IL2 fusion protein in mouse experimental system, as well as in initiated Phase I clinical trial with similar fusion protein of different specificity, hu14.18-IL2, administered to patients with GD-2 positive tumors.

1. The role of MHC class I expressed on the surface of tumor cells and NK cell involvement in KS-IL2 fusion protein induced killing in vitro :

Cytotoxicity assays performed in the presence of FP, using naive murine splenocytes as effectors and CT21.6 cells as targets, showed that killing of tumor cells occurs in a FP dose-dependent manner. Tumor cell line, CT26-Ep which express normal levels of MHC class I was killed at much lower level than the low class I expressor CT21.6. Upregulation of MHC class I on CT26.1 by in vitro IFN-γ treatment decreased the killing. This was associated with the level of induced MHC class I molecules on the surface of these tumor cells (Fig. 1). These results suggest that the in vitro killing of CT21.6 tumor cells mediated via KS-IL2 fusion protein involves NK cells and this activity can be inhibited by upregulation of MHC class I expression and also suggests the potential involvement of the killer inhibitory receptors on NK cells in this process (1,2). Furthermore they are a preliminary indication for choosing additional agents to be administered with immunocytokine during the treatment.

2. Evaluation of the immune cells in patients during the ch14.18-IL2 fusion protein treatment:

We evaluated patients who received 4 hour infusions of the fusion protein on days 1,2 and 3 every 28 days (one course of treatment). Decreased lymphocyte count during the treatment was observed from day 1 through day 4 and then increased significantly starting from day 5 and continuing to the end of the first course of treatment (day 22) (Fig. 2). These findings are similar to our previous experience with clinical trials involving IL-2 administration (3). Flow cytometric technique was used also for analysis of surface markers on these cells. Significant expansion of

CD16 and CD56 cells was observed on day 8 of treatment in comparison to day 1 (Fig. 3). These findings suggest stimulatory influence of fusion protein administration on immune cells of treated patients (4).

3. Measurement of the serum level of circulating fusion protein in patients during therapy:

Specific enzyme-linked immunosorbent assay (ELISA) method was used for evaluation of serum specimens from patients subjected to ch14.18-IL2 fusion protein therapy. By this sensitive assay we are able to measure fusion protein concentration as low as 0.2 ng/ml with accuracy > 95% (5). Multiple serum samples were collected from patients during the treatment. In the evaluated specimens the peak serum level of fusion protein was observed immediately after the end of infusion (4 hrs), and fusion protein was detectable through 16 hrs after infusion started. The concentration of fusion protein in serum was dependent on the dose administered to the patients (Fig. 4).

4. Assessment of functional activity of hu14.18-IL2 fusion protein present in patients circulation during therapy:

Serum specimens obtained from patients subjected to fusion protein therapy were evaluated by flow cytometry to assess ability of fusion protein remaining in these serum samples to bind to GD-2 positive cells. LAN-5 neuroblastoma cell line and M-21 melanoma cell line were used for these experiments. Binding of fusion protein to the cell surface was detected by antibody recognizing human IL-2. In these experiments serum samples taken from patients during fusion protein administration showed significant fluorescence signal, which indicated that fusion protein present in these specimens retaining its binding ability to GD-2 disialoganglioside present on the cell surface of control cell lines. Also integrity of the molecule is intact, because fusion protein bound to these cells is detected by antibody against IL-2 component of this fusion protein (Fig. 5).

5. Cytokine component of fusion protein present in patient serum during the treatment is active :

Serum specimens obtained from patients on fusion protein treatment were used to

stimulate IL-2 responsive cell line in a 72 hrs cell proliferation assay. Tf-1β cells, created in our lab, which express intermediate IL-2 receptor, are able to respond to IL-2 which results in ^{3}H-thymidine incorporation (6). In this experiments, cells were proliferating in the presence of serum taken from patients in different times during fusion protein therapy. This results indicate, that IL-2 component of fusion protein circulating in patient serum is retaining its stimulatory activity. Patients specimens acted as a source of IL-2 for an IL-2 responsive cell line. This activity was detected immediately after starting of the fusion protein infusion and remained for several hours after the end of fusion protein administration (Fig. 6).

6. Fusion protein present in patients serum facilitates ADCC in vitro with effector cells from normal donor :

Serum samples obtained from patients before starting fusion protein therapy as well as at the end of the 4 hrs of hu14.18-IL2 infusion were evaluated in a ^{51}Cr release assay (7). PBMCs obtained from a healthy donor were used as effectors and GD-2 positive LAN-5 cells were used as targets. Specimens obtained at the end of hu14.18-IL2 infusion, representing the highest level of fusion protein were able to facilitate significantly higher ADCC in this *in vitro* system, than specimens obtained prior to the infusion (Fig. 7). This result indicate that serum from patients during fusion protein therapy can serve as a source of antibody for ADCC and further that the fusion protein present in patient circulation elicits ADCC on normal effector cells.

7. PBMCs obtained from patients completed 3 infusions of hu14.18-IL2 fusion protein are more potent in facilitating ADCC in vitro :

To evaluate the influence of hu14.18-IL2 fusion protein treatment on the ability to facilitate ADCC by patient immune cells a ^{51}Cr release assay was used. PBMCs obtained from patients just before initiating of the fusion protein treatment as well as 5 days after the 3rd infusion were used as effector cells. In comparison to the pretreatment PBMCs, cells obtained after fusion protein treatment were able to kill much more efficiently LAN-5 targets in the presence of fusion protein in the assay medium (Fig.8). This result indicates activation of patient's immune cells by fusion protein therapy.

Summary of the significance of the related work :

The preliminary data described above showed that is possible to safely administer fusion protein to the cancer patients, this fusion protein remains stable in the circulation for several hours without loosing its functional activity and we are able to detect this molecule in obtained specimens. Presented work is based on GD-2 expressing tumors which represent a minority of human cancers. However, the principles which will emerge from this series of studies is likely to have broad applicability to antibody-based targeting of human cancers in general. Findings from studies of the hu14.18-IL2 fusion protein administration as a single agent and in the near future in conjunction with other molecules, can readily be applied to other tumor models using immunocytokines involving antibodies directed at specific tumor targets. Further, *in vitro* and preclinical studies, as well as clinical trials will elucidate some of the mechanisms involved in enhancing immune effectors against tumor cells, and these principles, too, will be broadly applicable. The future clinical trials designed based on the results of pre-clinical and *in vitro* experiments will provide a model for bringing novel immunocytokines to the clinic, and hopefully will improve effectiveness of the treatment of human cancers.

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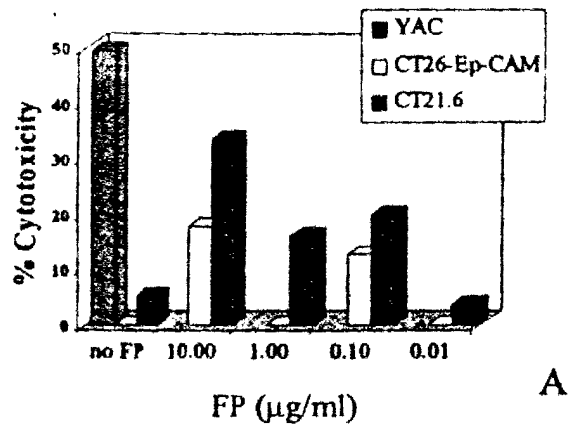
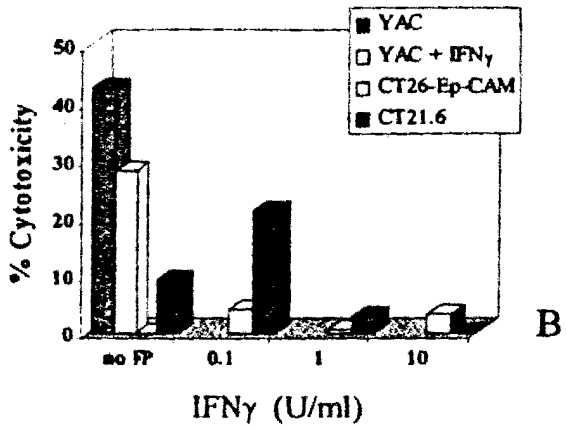
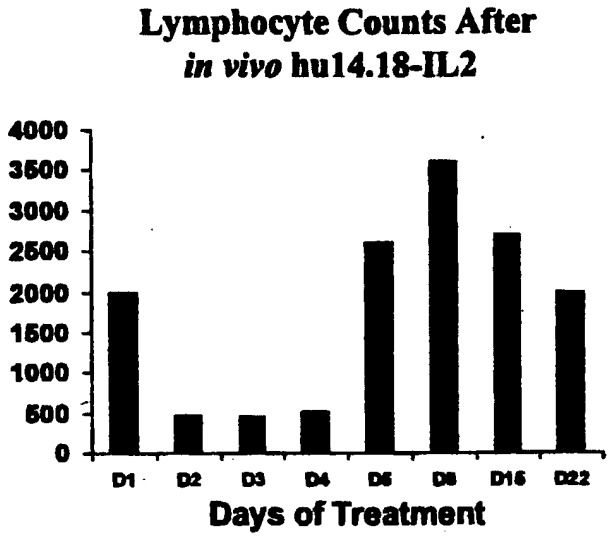
FIGURE 1:





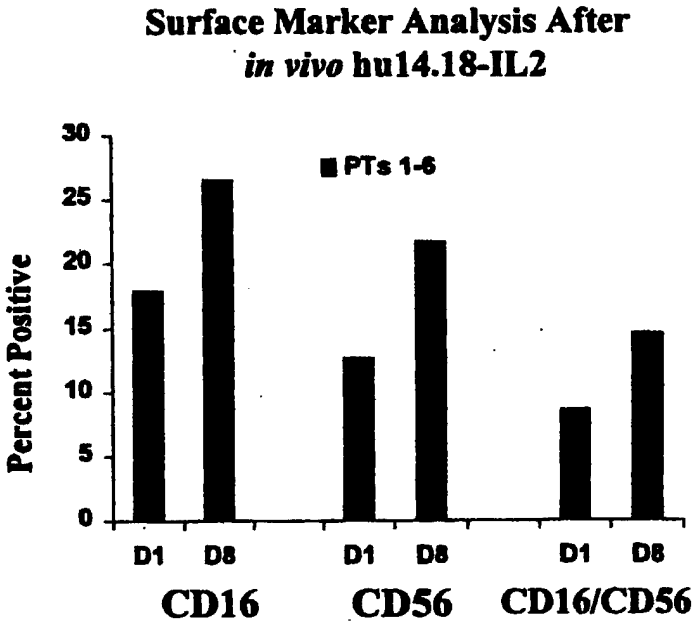
FP and MHC class I dependent killing of Ep-Cam positive tumor cells. A, Splenocytes from poly I:C stimulated mice were used as effector cells in ^{51}Cr killing assays against either CT26-Ep or CT21.6 targets and in the presence of various amounts of KS-IL-2 FP. B, splenocytes were used as effectors against either CT26-Ep or CT21.6 target cells that were cultured at different concentrations of IFNγ for 48 hrs prior to the assay. YAC cells were used as a positive control for NK mediated killing in the absence of FP in both assays. The FP concentration used in assay B was 10μg/ml.

FIGURE 2:



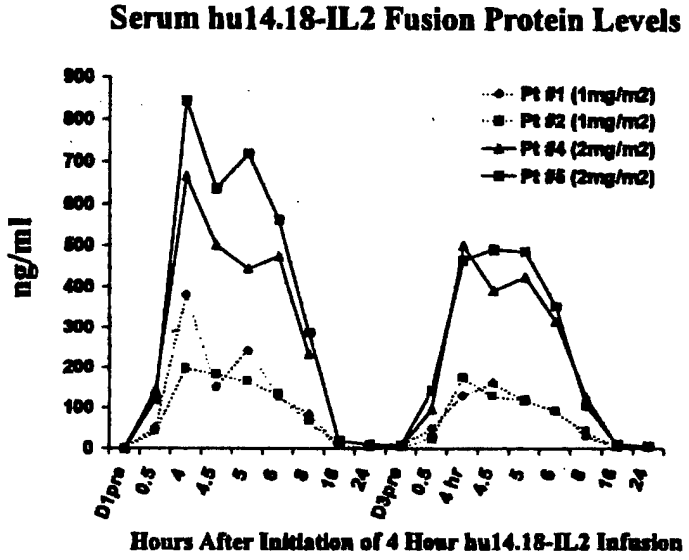
One representative patient's lymphocyte counts are shown above. This patient received 2 mg/m²/d of hu14.18-IL2 on Day 1, 2 and Day 3. A lymphopenia is seen following the Day 1 infusion and continues through Day 4, followed by an increase in lymphocyte counts on Day 5, 8, and 15. When values from the first 6 patients are examined, the values on Days 2, 3, and 4 are all less than day 1 ($p<0.0003$), and the value from Day 8 is greater than from Day 1 ($p<0.005$).

FIGURE 3:



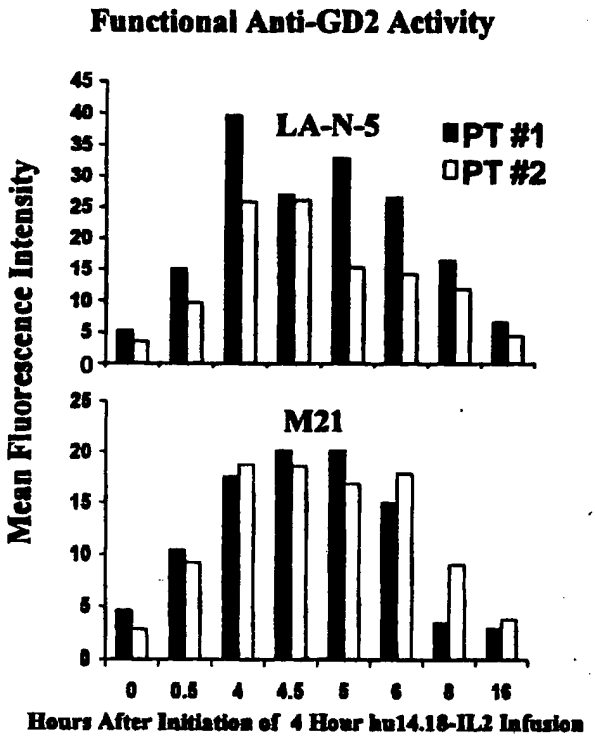
Flow cytometry results showing expansion of CD16 and CD56 cells, following *in vivo* treatment with hu14.18-IL2. The results shown are mean values for the first 6 patients' PBL obtained just before any treatment (D1) and on Day 8. The differences between Day 1 and Day 8 values for each marker were statistically significant. The p value for CD16 was 0.022; for CD56, p = 0.002; and for CD16/CD56, p = 0.003

FIGURE 4:



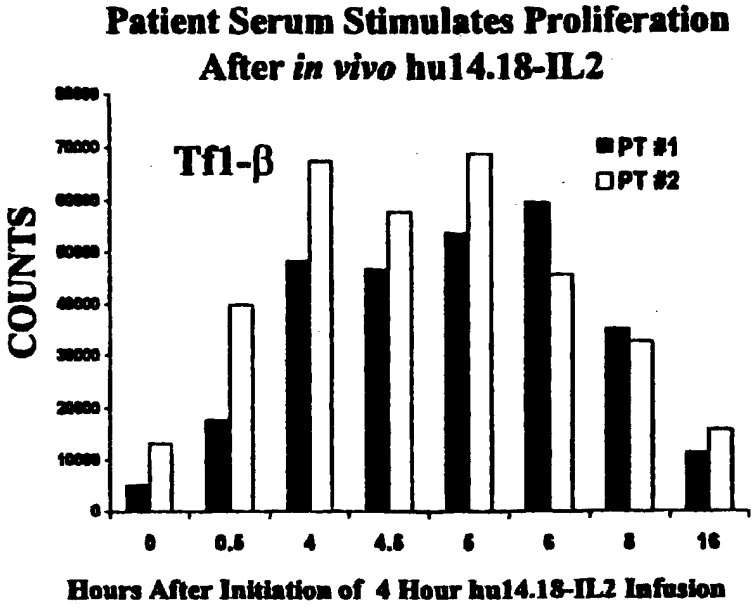
Serum samples obtained from patients at various timepoints were assayed in ELISA quantitating intact fusion protein via capture of the anti-id and development with anti-IL2 antibody. On Day 1 and Day 3 control (pre) samples were taken before 4 hrs infusion of fusion protein. Subsequent timepoints are indicated in hours after the start of the infusion.

FIGURE 5:



Serum obtained from 2 patients who received 1 mg/m² of fusion protein was used in an indirect flow cytometry staining assay on GD2+ cell lines, LA-N-5 and M21. Rat anti-human IL2 conjugated to phycoerythrin was the secondary reagent. The results show that the serum of these patients contains fusion protein that is able to bind to the tumor cells and that the IL2 portion of the fusion protein can be detected, bound to these cells.

FIGURE 6:



Serum obtained from patients who received 1mg/m² was used as the source of fusion protein in a 72 hr proliferation assay. Tf1-β cells, an IL2 responsive cell line, responds to the IL2 of the fusion protein found in patient serum at the various timepoints shown above. [The samples that show maximum proliferation were diluted out in further experiments (not shown) and did show the expected dilution effect.] Maximal proliferation is seen at 4 to 5 hours after initiation of the 4 hour infusion. The "0" timepoint is just before giving fusion protein and reflects background ^{3}H-TdR in this assay.

FIGURE 7:

Patient Serum After *in vivo* hu14.18-IL2 Ellicits ADCC on Normal Effectors

	Serum obtained			
	Day 1 pre infusion		Day 1- 4hrs (at stop of infusion)	
Serum Source	Lytic Units	% Cytotoxicity	Lytic Units	% Cytotoxicity
Patient 1	20	7	73	30
Patient 4	63	28	93	57
Patient 5	55	23	101	40

PBMC obtained from a healthy control donor were used as effectors in a 4hr ^{51} Cr release assay using serum obtained from patients receiving fusion protein at 1mg/m^2(patient 1) and 2 patients receiving fusion protein at 2mg/m^2, as a source of antibody for ADCC. The data are expressed as lytic units per 10^6 effectors achieving a 20% lysis of $5x10^3$ LA-N-5 targets. The % cytotoxicity is expressed at a 50:1 effector to target ratio. Patient serum was obtained just prior to starting (pre) and just at the completion of the 4hr hu14.18-IL2 infusion.

FIGURE 8:

Patient PBMC Ellicit ADCC After *in vivo* hu14.18-IL2

PBMC obtained from	Lytic Units on LA-N-5 target in medium supplemented with		
	medium	IL2	Fusion Protein
Pt 4 day 1	0	0	121
Pt 4 day 8	17	44	715
Pt 5 day 1	2	17	153
Pt 5 day 8	18	27	1838
Pt 6 day 1	4	11	396
Pt 6 day 8	8	40	548

PBMC obtained from 3 patients prior to initiating fusion protein on day 1, and on day 8, 5 days after the 3rd infusion, were used as effectors in a ^{51}Cr release assay. The assay was run in standard tissue culture medium supplemented with 10% human serum(medium), medium supplemented with IL2 at 100 units/ml, or medium supplemented with fusion protein at 0.25 μg/ml. The data are expressed in lytic units.