Vol. 189, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1992 Pages 92-100

Multidrug Resistant HOB1 Lymphoma Cells Express P-glycoprotein That Does Not Play the Major Role in the Development of Drug Resistance to Adriamycin

Wei-Ping Lee[1], Chao-Lin Lee, and Ming-Ching Kao

Department of Biochemistry, P. O. Box 90048-501, National Defense Medical Center, Taipei, Taiwan, R.O.C.

Received August 3, 1992

Summary. Two cell lines resistant to 0.1 μM vincristine (VCR) and 2.0 μM adriamycin (ADR), respectively, (designated HOB1/VCR0.1 and HOB1/ADR2.0) were established from a human immunoblastic B lymphoma cell line. These cell lines showed the typical MDR phenotype with overexpression of P-glycoprotein and decreased [^{3}H]VCR accumulation. The retention amounts of intracellular [^{3}H]VCR in these two cell lines could be augmented by verapamil. However, in spite of the overproduction of P-glycoprotein, both HOB1/VCR1.0 and HOB1/ADR2.0 cells did not exhibit decreased accumulation of intracellular [^{14}C]ADR. And the retention of [^{14}C]ADR was not affected by verapamil. Our data support that P-glycoprotein is a drug transporter more important for the development of drug resistance to VCR than to ADR.

Introduction. Both VCR and ADR have been widely used in cancer chemotherapy. The cell lines resistant to these two agents often display the multidrug-resistant phenotype (MDR) demonstrating cross-resistance to many structurally unrelated drugs (1). Resistance to these drugs can be overcome by various agents which competitively inhibit P-glycoprotein-mediated drug efflux, including the calcium channel blocker verapamil (2).

In certain clinical cases, an increase in P-glycoprotein can be detected in tumor cells from patients who have relapsed during treatment whereas

[1]To whom correspondence should be addressed.

The abbreviations: PBS, phosphate buffered saline, 136 mM NaCl, 2.5 mM KCl, 6.6 mM Na_2HPO_4, and 1.5 mM KH_2PO_4; SSC, standard saline citrate, 0.15 M sodium chloride, 0.015 M sodium citrate; SDS, sodium dodecyl sulfate; VCR, vincristine; ADR, adriamycin; MDR, multidrug resistance.

0006-291X/92 $4.00

in certain other similar cases, there is no evidence for the presence of this protein (3, 4). Some investigators have isolated non-P-glycoprotein-mediated multidrug resistant cell lines (5, 6). It seems that discrete mechanisms induced by resistant cells for different agents play even more important roles than P-glycoprotein. For ADR, free radical scavengers such as glutathione S-transferase and glutathione peroxidase can be over-expressed in some resistant cells (7, 8).

In the present study, we show P-glycoprotein overexpressed in either VCR- or ADR-resistant HOB1 lymphoma cells does not confer lower ADR accumulation in contrast to previously reported cell lines (9-11).

Materials and Method

Materials. Chemotherapeutic agents including VCR and ADR, verapamil, and MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) were purchased from Sigma. [^{3}H]VCR, [^{14}C]ADR, Klenow enzyme for probe labeling and sheep anti-mouse IgG were supplied by Amersham. JSB-1 monoclonal antibody was provided by Caltag Laboratories.

Cell lines and Cell culture. HOB1 lymphoma cells were cultured as described previously (12). Drug-resistant cell lines were established by stepwise increasing the drug doses to 0.1 μM VCR for HOB1/VCR$_{0.1}$ cells and 2.0 μM ADR for HOB1/ADR$_{2.0}$ cells, respectively.

Western blot analysis for P-glycoprotein. A membrane fraction containing both plasma membrane and endoplasmic reticulum was isolated as described previously (13). Polyacrylamide gel electrophoresis was performed according to the method of Laemmli (14). Immunoblottings were performed as described previously (15).

Northern blot analysis for the expression of *mdr*1 gene. The SalI + BamHI-generated 4.3 Kb cDNA probe of human *mdr*1 was labeled by random priming method (16). Total RNA was purified by acid guanidium thiocyanate-phenol-chloroform extraction (17). 20 μg total RNA of each cell line was fractionated by 1% agarose gel containing 6.7% formaldehyde. RNA was transferred to Hybon-N filters, uv crosslinked, and hybridized at 42° C for 24 hours in hybridization buffer (6 x SSC, 50% deionized formamide, 10 x Denhardt's solution, 10 mM EDTA, and 0.1% SDS) containing 1 x 10^6 cpm of labeled probe per ml. After hybridization, the filters were washed in 2 x SSC and 0.1% SDS with a final wash in 0.1 x SSC and 0.1% SDS at 60° C, followed by exposure on Kodak XAR film. The same blots were washed in 0.1 x SSC and 0.1% SDS at 80° C for 4 hours and then reprobed with ^{32}P-labeled human β-actin cDNA.

In vitro cytotoxicity assays. Exponentially growing cells were harvested, washed, and resuspended in an appropriate amount of medium with 1 x 10^5 cells/ml. Cells were seeded into 96-well microtiter plates at 20,000 cells/well in 0.1 ml of medium containing increased concentration of drugs with replicates of eight. After incubation at 37° C for 96 hours, the treated cells were processed for MTT assays (18). Fold resistance was determined

by the ratio of the IC_{50} (the drug concentration causing 50% cell killing) of drug-resistant to parental sensitive HOB1 cells.

Drug uptake studies. Aliquots of 1 x 10^6 cells were harvested, washed and resuspended with 0.5 ml of PBS containing 0.1 μM [^{3}H]VCR (6.5 Ci/mmol) or 0.5 μM [^{14}C]ADR (55 mCi/mmol) with or without 10 μM verapamil added. At specific intervals, labeled cells were layered on 0.2 ml of a silicon oil: mineral oil mixture (4:1, v/v) and centrifuged 10 sec. at 15,000 x g. The cell pellets were washed with cold PBS. Tritium activity was measured in 4 ml of cocktail with a liquid scintillation counter after the pellets were dissolved in 0.1 ml of 0.1 N NaOH.

Drug efflux studies. Aliquots of 1 x 10^6 cells were harvested, washed and resuspended in 0.5 ml of PBS with 0.1 μM [^{3}H]VCR or 0.5 μM [^{14}C]ADR added. After incubation for one hour at 37° C , the cells were centrifuged at 1000 x g, and washed with PBS to remove extracellular radioactive agents. The cell pelletes were resuspended in 0.5 ml of PBS and continued to be incubated at 37° C. At specific intervals, the cells were pelleted by centrifugation at 15,000 x g for 10 sec. and washed twice with cold PBS. Tritium activity was determined as described in drug uptake studies. The data of the intracellular drug retention were expressed as a percentage of the initial intracellular drug concentration.

Verapamil reversal tests. HOB1/ADR$_{2.0}$ cells exposed to 0.5, 1.0, and 2.0 μM ADR, respectively, were treated with increased concentrations of verapamil. For HOB1/VCR$_{0.1}$, the cells maintained in 0.01, 0.05, and 0.1 μM VCR, respectively, were treated with increased concentrations of verapamil. After 4-day incubation, the viable cells were tested using MTT assays (18). The survival fractions were expressed as the ability of tetrazolium reduction of treated cells over that of untreated cells.

Results and Discussion

Establishment of drug-resistant cell lines. The morphology of both HOB1/VCR$_{0.1}$ and HOB1/ADR$_{2.0}$ cells was similiar to that of parental cells. ADR in stocks was scarlet. The pellets of viable HOB1/ADR$_{2.0}$ cells showed a pink color which indicated a large amount of ADR was retained within the resistant cells (Fig. 1). The accumulated adriamycin must be modified, or the cells would be killed by free radicals induced by the intact drug. Alternatively, free radical scavengers might have to overwork to protect the cells from the injury of free radicals.

Cytotoxic effect of VCR and ADR in resistant and parental cells. HOB1/VCR$_{0.1}$ cells showed 310-fold resistance to VCR but only 2.5-fold to ADR (Table 1). The MDR cells are usually more resistant to the selective agent than to other hydrophobic agents. Interestingly, HOB1/ADR$_{2.0}$ cells showed 15.7-fold resistance to ADR but 320-fold to VCR. Verapamil enhanced the cytotoxicity of VCR to a much greater extent than that of ADR. HOB1/VCR$_{0.1}$ cells showed no significant difference in fold

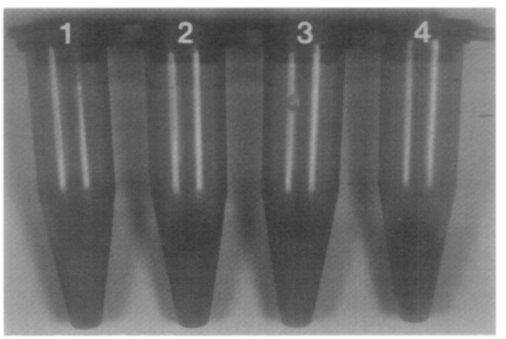


Figure 1. Resistant cell pellets of pink color observed in blue microcentrifuge tubes. 1. HOB1/parent; 2. HOB1/ADR0.5; 3. HOB1/ADR1.0; 4. HOB1/ADR2.0.

resistance from parental cells when incubated in the presence of 10 μM verapamil. Table 2 exhibited the cytotoxic effects of VCR and ADR on HOB1/ADR0.5, HOB1/ADR1.0 and HOB1/ADR2.0 cells resistant to 0.5, 1.0, and 2.0 μM ADR, respectively. HOB1/ADR1.0 and HOB1/ADR2.0 cells though showing different degrees of resistance to ADR had no significant difference in resistance to VCR.

Analyses of P-glycoprotein and *mdr*1 gene. As previously reported cell lines (9-11), both HOB1/VCR0.1 and HOB1/ADR2.0 cells overproduced P-glycoprotein (Fig. 2) and showed increased expression of *mdr*1 gene (Fig. 3A). The expression amounts of *mdr*1 gene in these two resistant cell lines approached to approximately the same level (Fig. 3A) This was consistent with the fold-resistance of HOB1/VCR0.1 and HOB1/ADR2.0 cells to VCR.

Table 1. Comparisons of the cytotoxicity of VCR and ADR in parental, HOB1 /VCR0.1 and HOB1/ADR2.0 cells. The data in parentheses represented fold resistance of resistant cells compared with parental cells. The average error was about 15%.

	IC_{50} (μM)[a]		
Agents	HOB1	HOB1/VCR0.1	HOB1/ADR2.0
Vincristine	0.0020	0.62(310)	0.64(320)
Adriamycin	0.35	0.86(2.5)	5.51(15.7)
Vincristine(V)[b]	0.0020	0.0018(0.9)	0.0023(1.2)
Adriamycin(V)[b]	0.38	0.35(0.92)	1.61(4.6)

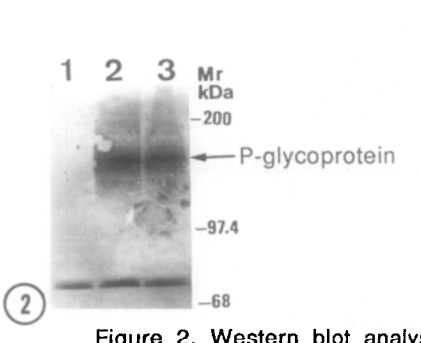
[a] The drug concentrations causing 50% cell killing.
[b] MTT assays were performed with 10 μM verapamil added.

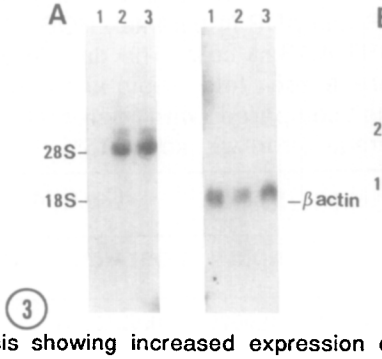
Table 2. The cytotoxic effects of VCR and ADR on various levels of resistance of HOB1 cells to ADR. The data in parentheses represented fold resistance as compared with the parental cells (refer to Table 1). The average error was about 15%.

	IC_{50} (μM)		
Agents	HOB1/ADR0.5	HOB1/ADR1.0	HOB1/ADR2.0
Vincristine	0.56 (280)	0.58(290)	0.64(320)
Adriamycin	2.20 (6.3)	2.22(6.3)	5.51(15.7)

There was no significant increase in the expression of *mdr*1 gene when the levels of resistance rose from HOB1/ADR0.5 to HOB1/ADR2.0 (Fig. 3B).

Kinetics of drug transport in drug-resistant and -sensitive cells. Amounts of drug uptake were expressed as a function of time. Fig. 4A demonstrated HOB1/VCR0.1 cells retained approximately 2.3 fold less [^{3}H]VCR than parental cells. The accumulation of [^{3}H]VCR was accelerated by verapamil in HOB1/VCR0.1 cells, but was not affected in parental cells (Fig. 5). Unexpectedly, HOB1/ADR2.0 cells revealed approximately 2 fold higher accumulation of [^{14}C]ADR as compared with parental and HOB1/VCR0.1 cells (Fig. 4B). Moreover, verapamil had no significant effect on the accumulation of [^{14}C]ADR (Fig 5).





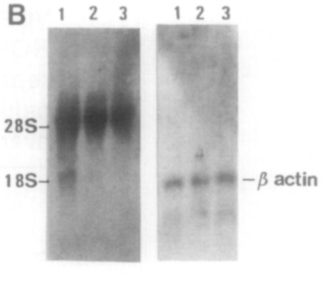


Figure 2. Western blot analysis showing increased expression of P-glycoprotein in resistant cells. 1. HOB1/parent; 2. HOB1/VCR0.1; 3. HOB1/ADR2.0.

Figure 3. Northern blot analyses showing the expression of *mdr*1 gene. A: Increased expression of *mdr*1 gene in the resistant cells. 1. HOB1/parent; 2. HOB1/VCR0.1; 3. HOB1/ADR2.0. B: The expression of *mdr*1 gene was not enhanced by increased levels of adriamycin resistance. 1. HOB1/ADR0.5; 2. HOB1/ADR1.0; 3. HOB1/ADR2.0.

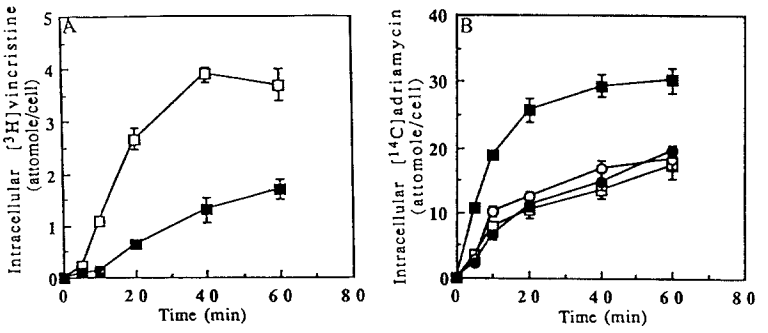


Figure 4. Time course of the uptake of radioactive agents in parental and resistant cells. A: [^{3}H]VCR uptake of HOB1/parent (□) and HOB1/VCR0.1 (■) cells in 60 minutes. B: [^{14}C]ADR uptake of HOB1/parent (□), HOB1/ADR2.0 (■), HOB1/ADR1.0 (○), and HOB1/ADR0.5 (●) cells in 60 minutes.

Drug efflux was expressed as the percentages of retention amounts in the initial level plotted versus specific times. Fig. 6 displayed greater [^{3}H]VCR efflux in HOB1/VCR0.1 cells (58% efflux of the initial level in contrast to 13% in parental cells at 60 minute). However, HOB1/ADR2.0 cells showed even slower [^{14}C]ADR efflux than parental cells (52% of the initial level remaining in resistant cells compared with 36% in parental cells). Slower ADR efflux may lead to higher drug retention in HOB1/ADR2.0 cells. HOB1/ADR0.5 and HOB1/ADR1.0 cells did not show significant difference from parental cells in the uptake and efflux of [^{14}C]ADR.

We speculate that there may be two strategies for HOB1/ADR2.0 cells to resist adriamycin. The one is P-glycoprotein which drives the agent out of the cells, and the other is the intracellular organelles which trap the agent, and by unknown mechanisms render it ineffective in killing the cells. The latter effect should be much greater than that of the former in HOB1/ADR2.0 cells. In view of the pink cell pellet of HOB1/ADR2.0 cells, we

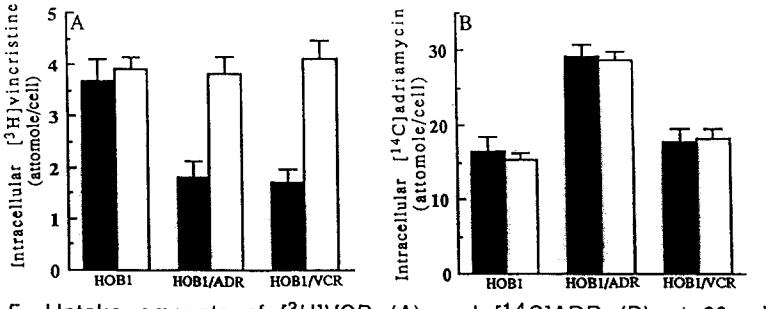


Figure 5. Uptake amounts of [^{3}H]VCR (A) and [^{14}C]ADR (B) at 60 minute in HOB1/parent, HOB1/VCR0.1 and HOB1/ADR2.0 cells with (open bars) or without (closed bars) 10 μM verapamil added.

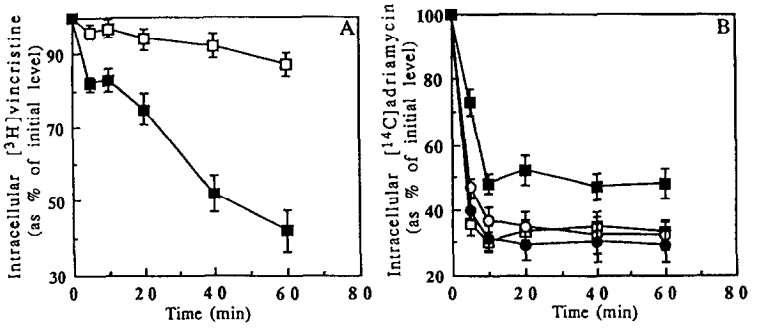


Figure 6. Efflux of radioactive agents in parental and resistant cells. A: Efflux of [^{3}H]VCR in HOB1/parent (□) and HOB1/VCR0.1 (■) cells. B: Efflux of [^{14}C]ADR in HOB1/parent (□), HOB1/ADR2.0 (■), HOB1/ADR1.0 (○), and HOB1/ADR0.5 (●) cells.

think the higher the level of resistance, the stronger the effect of the latter. Moreover, the expression of P-glycoprotein did not increase in direct proportion to the level of resistance. The drug-efflux function of P-glycoprotein in HOB1/VCR2.0 cells is offseted by the overwhelming trapping effect. This effect lacking induced detoxifing enzymatic systems may also be present intrinsically in parental cells and so it shades the pumping effect of P-glycoprotein expressed in resistant cells. The net effect in difference between parental and lower level resistant cells is beyond detection by conventional methods, but is unveiled in HOB1/ADR2.0 cells. Deffie *et al.* have demonstrated an active extrusion pump was not operational in cloned ADR-resistant P388 leukemia cells that had P-glycoprotein expression (7). Our data also give evidence that a active role for the P-glycoprotein serving as a drug efflux pump for ADR is not prominent in HOB1/ADR2.0 cells.

Verapamil reversal tests. Fig. 7A demonstrated resistant cells incubated in 0.5 μM ADR were refractory to verapamil reversal; however, the cells

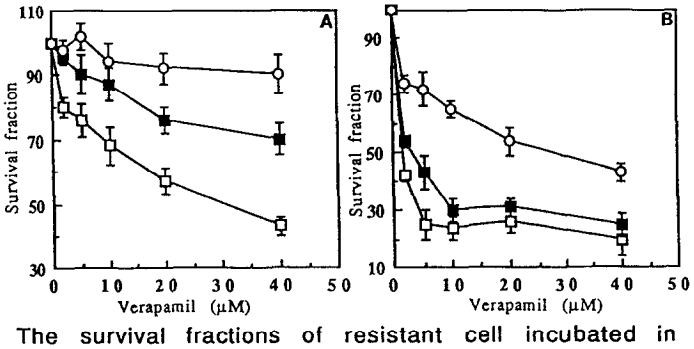


Figure 7. The survival fractions of resistant cell incubated in increased concentrations of verapamil. A: HOB1/ADR2.0 cells were incubated in 0.5 (○), 1.0 (■), and 2.0 μM (□) ADR. B: HOB1/VCR0.1 cells were incubated in 0.01 (○), 0.05 (■), and 0.1 μM (□) VCR.

Table 3. Effect of verapamil on resistant HOB1 cells with different levels of resistance to ADR. Each cell line of different levels of resistnce, maintained in 0.5, 1.0, and 2.0 μM ADR, respectively, was treated with increased concentrations of verapamil (μM). After 4-day incubation, the living cells were processed for MTT assays. The IC_{50} referred to the concentrations of verapamil causing a 50% decrease of tetrazolium reduction.

	IC_{50}(μM)		
Adriamycin (μM)	HOB1/ADR0.5	HOB1/ADR1.0	HOB1/ADR2.0
0.5	> 100	> 100	> 100
1.0	52 ± 3.4	63 ± 4.5	72 ± 4.9
2.0	15 ± 1.2	17 ± 2.3	22 ± 1.6

incubated in 2.0 μM ADR were sensitive to increased concentrations of verapamil. On the other hand, we also compared the servoeffect of verapamil on enhancing the cytotoxicity of ADR in HOB1/$ADR_{0.5}$, HOB1/$ADR_{1.0}$ and HOB1/$ADR_{2.0}$ cells (Table 3). These cells were adequate to deal with the loading of 0.5 μM ADR. Once they were incubated in 2.0 μM ADR, they became sensitive to verapamil. Verapamil may interfere with the enzymatic systems which render the cytotoxic agents ineffective. Its competitive inhibition of P-glycoprotein function may indirectly reinforce this effect. Fig. 7B exhibited HOB1/$VCR_{0.1}$ cells were extremely sensitive to verapamil reversal even if the concentration of VCR was lowered to 0.01 μM.

HOB1/$ADR_{2.0}$ cells established here will be an useful material for studying the mechanisms of resistance of tumor cells to ADR. The data presented in this paper give an implication that P-glycoprotein detected in clinical drug-resistant tumor cells does not represent this protein plays the major role in the development of drug resistance.

Acknowledgments. The authors thank Dr. Ho Y-S for kindly providing HOB1 lymphoma cell line and Dr. Chao C-K for human *mdr*1 cDNA.

References

1. Endicott, J. A., and Ling, V. (1989) Ann. Rev. Biochem. 58, 137-171.
2. Cornwell, M. M., Pastan, I., and Gottesman, M. M. (1987) J. Biol. Chem. 262, 2166-2170.

3. Bell, D. R., Gerlach, J. H., Kartner, N., Buick, R. N., and Ling, V. (1985) J. Clin. Oncol. 3, 311-315.
4. Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M., and Pastan, I. (1987) Proc. Natl. Acad. Sci. USA 84, 265-269.
5. Harker, W. G., Slade, D. L., Dalton, W. S., Meltzer, P. S., and Trent, J. M. (1989) Cancer Res. 49, 4542-4549.
6. Cole, S. P. C., Chanda, E. R., Dicke, F. P., Gerlach, J. H., and Mirski, S. E. L. (1991). Cancer Res. 51, 3345-3352.
7. Deffie, A. M., Alam, T., Seneviratne, C., Beenken, S. W., Batra, J. K.,Shea, T. C., Henner, W. D., and Goldenberg, G. J. (1988) Cancer Res. 48, 3595-3602.
8. Sinha, B. K., Mimnaugh, E. G., Rajagopalan, S., and Myers, C. (1989) Cancer Res. 49, 3844-3848.
9. Inaba, M., Kobayashi, H., Sakurai, Y., and Johnson, R. K. (1979) Cancer Res. 39, 2200-2203.
10. Tsuruo, T., Iida, H., Tsukagoshi, S., and Sakurai, Y. (1982) Cancer Res. 42, 4730-4733.
11. Kartner, N., Shales, M., Riordan, J. R., and Ling, V. (1983) Cancer Res. 43, 4413-4419.
12. Ho, Y-S., Sheu, L-F., Ng, J. A., and Hsu, S-M. (1990) Br. J. Cancer 61, 655-658.
13. Garman, D., Albers, L., and Center, M. S. (1983) Biochem. Pharmacol. 32, 3633-3637.
14. Laemmli, U. K. (1970) Nature 227, 680-685.
15. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
16. Fernberg, A. P., and Vogelstein, B. A. (1983) Anal. Biochem. 132, 6-13.
17. Chomczynski, P., and Sacchi, N. S. (1987) Anal. Biochem. 162, 156-159.
18. Mosmann, T. (1983) J. Immunol Methods 65, 55-63.