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COEXPRESSION OF CYTOCHROME P4502A6 AND HUMAN NADPH-P450 OXIDOREDUCTASE IN THE BACULOVIRUS SYSTEM

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ABSTRACT:

Heterologous expression using baculovirus vectors has become a popular method for the production of catalytically active cytochrome P450s (CYPs). We have systematically optimized the multiplicity of infection (MOI) for a coinfection approach for the coexpression of CYP2A6 (viral vector designated v2A6) and NADPH-P450 oxidoreductase (OR; viral vector designated vOR) using Sf9 insect cells. A 3000-fold range of MOI was examined in stationary culture and stirred suspension culture. Surprisingly, our results indicate that the best CYP2A6 catalytic activity (850–1300 pmol/min/mg total lysate protein as measured by coumarin 7-hydroxylase activity) was obtained only when using a low MOI of v2A6 ($1.5–3 \times 10^{-2}$) and a vOR of 10- to 20-fold less. This activity was ~7- to 11-fold higher than the best activity obtained when infecting cells with v2A6 alone. At this level of coinfection, the P450 content ranged from 180 to 250 pmol/mg total lysate protein, and the NADPH cytochrome *c* reductase activity ranged from 350 to 520 nmol/min/mg total lysate protein. Increasing the MOI of both viruses to 50-fold higher resulted in lower overall activity with the optimum (250 pmol/min/mg total lysate protein) being seen earlier postinfection (60 *vs.* 72 hr). Increasing the MOI of vOR to levels comparable with those of v2A6, decreased coumarin 7-hydroxylase activity 14-fold. These results suggest that the best CYP2A6 catalytic activity depends on properly posttranslationally modified proteins accumulating in a right ratio as a result of primary, secondary, and possibly tertiary infection of both viruses. These results also suggest that high OR expression results in degradation of P450.

CYPs[1] are a multienzyme, membrane-bound system that metabolizes many drugs and other xenobiotics (1). The catalytic activity of CYP enzymes requires the presence of NADPH-CYP OR. In addition, cytochrome b_5 stimulates catalytic activity for some CYP forms (2).

Several efficient systems for the heterologous expression of mammalian CYP enzymes have been developed, including bacterial, yeast, and mammalian and baculovirus/insect cell-based systems (reviewed in ref. 2). Of the systems available, the baculovirus system has distinct advantages for the production of high levels of active, native CYP enzyme. Although efficient mammalian CYP expression is possible in bacterial systems, modification of the amino acid sequence is usually required for high level expression (2).

Three distinct approaches have been taken for production of catalytically active CYP enzymes using baculovirus system. Expression of the CYP enzyme alone and then reconstitution with OR using purified P450s or total cell lysate (3–5), coexpression of the CYP and OR using a single virus (6), and coinfection with independent viruses containing CYP or OR (7). Each approach has unique advantages and disadvantages. Purification/reconstitution provides flexibility in controlling the CYP to OR ratio, but is time- and labor-intensive because of the need for column purifications. The single virus approach is more time- and labor-efficient (simple cell lysate can be used), but, because of the small number of promoters characterized for baculovirus expression, the ability to control the CYP to OR ratio is limited (6, 8). Coinfection has the potential to provide the ability to control CYP to OR ratio (by changing MOI for each virus) and also permits the use of simple cell lysate (7).

The human CYP2A6 is the only enzyme known to date responsible for coumarin 7-hydroxylase activity in human liver (9, 10). CYP2A6 also metabolically activates certain carcinogens and promutagens (for review, see refs. 1, 9, and 10). It has the highest activity for activation of the mutagen *N*-nitrosodiethylamine among all human P450s studied (9, 11). It also activates tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, 1-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-4-butanal, *n*-nitrosonornicotine (12, 13), and the mycotoxin aflatoxin B1 (14, 15).

In this study, we describe a systematic characterization of a coinfection approach using CYP2A6 and human OR. To obtain an overall phenomena for coexpression of P450 and OR proteins in the baculovirus system, we examined a wide range of MOI for both v2A6 and vOR, with different ratios for coinfection to determine the best ratio for optimal coumarin 7-hydroxylase catalytic activity in insect cell lysate.

Materials and Methods

Rabbit anti-rat 2A1 (16) and rabbit anti-rat OR (17) antibodies were described in the previous studies. Recombinant baculoviruses v2A6 and vOR

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[1] Abbreviations used are: CYP, cytochrome P450; OR, NADPH-P450 oxidoreductase; P450, cytochrome P450; MOI, multiplicity of infection (the number of virus used per cell); v2A6, a recombinant baculovirus containing the human CYP2A6 cDNA; vOR, a recombinant baculovirus containing the human OR cDNA; Sf9, *Spodoptera frugiperda*; pfu, plaque-forming units (measurement of infectivity of a virus); AcMNPV, *Autographa californica* nuclear polyhedrosis virus.

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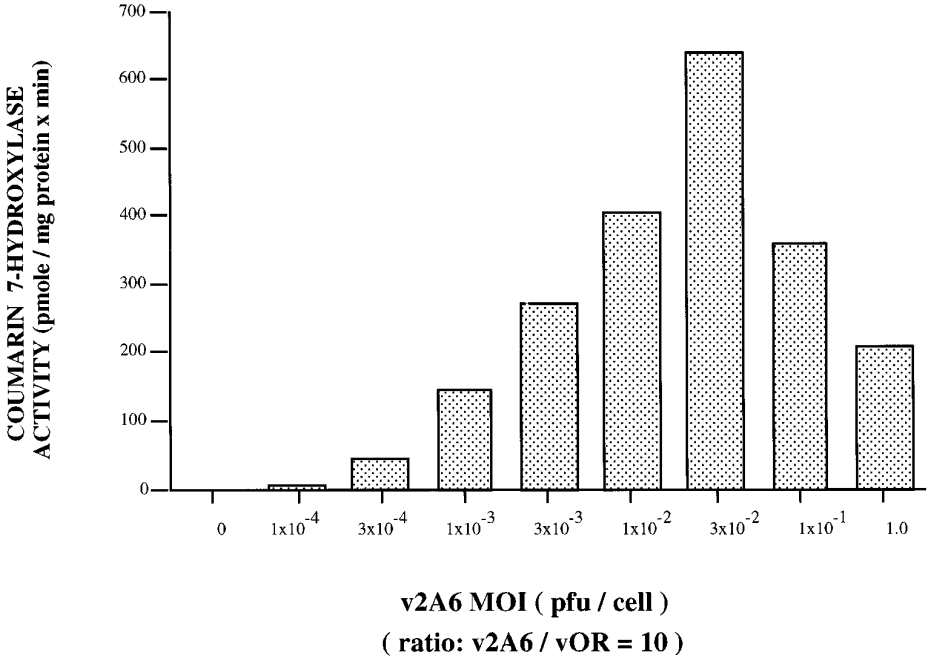

FIG. 1. *Effect of v2A6/vOR MOI (ratio 10) on coumarin 7-hydroxylase activity.*

Cells were coinfected with indicated pfu of v2A6 and 1/10 pfu of vOR, and incubated for 72 hr at 27°C. Hemin was added at 24 hr postinfection to 4 μg/ml as described (19). Coumarin-7-hydroxylase activity was assayed as described in *Materials and Methods.*

were also described in earlier studies (7). Briefly, both cDNAs were inserted in the pAc373 vector, and their expressions were controlled by the polyhedrin very late promoter (*polh*). Sf9 insect cells were purchased from the ATCC (Rockville, MD). Grace's insect media with L-glutamine, yeastolate, and lactalbumin supplements, and baculovirus agarose were from Invitrogen (San Diego, CA). Fetal bovine calf serum (heat inactivated) was purchased from Sigma Chemical Company (St. Louis, MO). Nitrocellulose membrane was obtained from Schleicher & Schuell (Keene, NH). Molecular biological reagents were obtained from New England Biolabs (Beverly, MA), and other reagents were obtained from Sigma Chemical Company.

Amplification of v2A6 and vOR Stocks and Virus Tittering. About 10 ml of each virus stock was used to infect 400 ml Sf9 cells at a density of 2×10^6 cells/ml in a 1 liter spinner flask. Virus stocks were harvested 7 days postinfection by centrifugation at 2500 rpm for 10 min to remove cells and debris. Stocks were tittered by plaque assay. Virus dilutions of 10^{-5} and 10^{-6} in 0.5 and 1 ml were used to infect 7×10^6 cells in 100 mm plates. Duplicate plates were used. Plaques were visualized by Neutral Red Stain 6 days postinfection as described (18). Briefly, agarose (5%) in distilled water was autoclaved and diluted to 0.5% with heated insect culture medium and cooled to 42°C. The stain was added to the 0.5% agarose to a final concentration of 50 μg/ml. About 5 ml of 0.5% stained agarose was used to overlay each plate. Plates were incubated at 27°C overnight. The titer of viral stocks was determined from plates containing 20–110 plaques/plate.

Production of Recombinant Proteins. Sf9 cells from the same culture were used for each set of experiments. In 24-well plates, 2.5×10^5 Sf9 cells in log phase were seeded into each well in 1 ml of complete insect culture medium prepared as described (Invitrogen). Sf9 cells were infected with v2A6 and vOR at the indicated MOI. Hemin at 2 mg/ml (in 50% ethanol and 0.2 M NaOH) was added 24 hr postinfection to a final concentration of 4 μg/ml as described (19). Cells were incubated at 27°C for 72 hr. To express enzyme in spinner flasks, log phase Sf9 cells were diluted to 1×10^6 cells/ml, and 80 ml of cells were used for each infection in a 100 ml spinner flask (or 200 ml of cells in 500 ml spinner flask). Cells were infected with the indicated virus(es); hemin was added as described. Cells were harvested at the indicated time, washed with 1 × phosphate-buffered saline, and resuspended in cell lysate buffer [0.1 M sodium phosphate (pH 7.4), 1.0 mM dithiothreitol, 1.0 mM EDTA, 20% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride] at a ratio of ~40 ml cultured cells to a 1.0 ml of cell lysate buffer, sonicated, and subjected to other studies.

Coumarin 7-Hydroxylase Activity Assay. For studies using 24-well plates, coumarin 7-hydroxylase activity was determined by adding 50 μl of 1.0 mM coumarin in Grace's media to each well containing 1 ml of infected cells 72 hr postinfection; fluorescence was measured after 1 hr incubation at 37°C using a Millipore Cytofluor 2350 with 360 and 460 nm filters and a bandwidth of 40 nm. The fluorescence signal-to-background ratio was increased by adding 0.5 ml of 0.1 M Tris (pH 9.0). For spinner flask infection, 10 μl of cell lysate was added to 490 μl of 0.1 M Tris (pH 7.5) containing 0.4 mM coumarin, 25 μg NADP+, and 0.3 units of glucose 6-phosphate dehydrogenase. The mixture was incubated at 37°C for 15 min. After incubation, 100 μl of reaction sample was transferred to 1.9 ml of 0.1 M Tris (pH 9.0) for fluorescence measurement as described (20).

Immunoblot. Total cell lysate proteins or microsomal proteins were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose filter as described (7). The filters were incubated overnight with an antibody mixture of 1:500 dilution of rabbit anti-rat 2A1 and 1:500 dilution of rabbit anti-rat OR. The blots were developed using 1:1500 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma Chemical Company) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

Other Methods. Cytochrome *c* reductase activity was determined as described (7). Spectrophotometric determination of P450 content was according to the method of Omura and Sato (21). Protein was determined by the bicinchoninc acid method (22), with a reagent kit obtained from Sigma Chemical Company.

Results

The MOI for v2A6 and vOR were systematically examined using CYP2A6-catalyzed coumarin 7-hydroxylase activity as an endpoint. Initial analyses were conducted in 24-well plates with *in situ* assay of the enzyme by addition of substrate to the wells and measurement of product using a fluorescent plate reader. Results were then extended to stirred suspension culture.

Determination of MOI of v2A6 and vOR for Coinfection. Sf9 cells were coinfected with v2A6 and vOR at a MOI of 5.0, 1.0, 0.2,

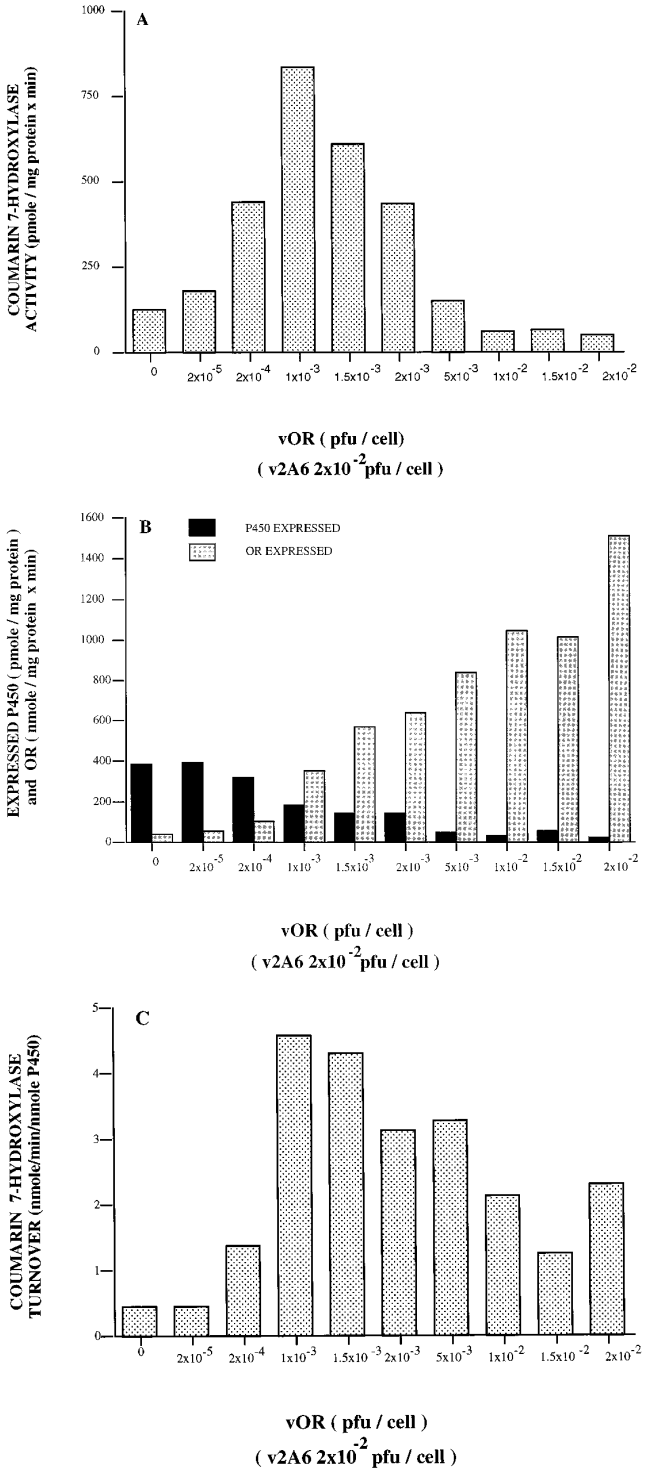

FIG. 2. *Effect of v2A6/vOR coinfection ratio on cell lysate for (A) coumarin 7-hydroxylase activity, (B) CYP2A6 P450 content and OR catalytic activity, and (C) enzyme turnover number.*

Cells were coinfected with 2×10^{-2} pfu/cell of v2A6 and the indicated pfu of vOR. Hemin was added at 24 hr postinfection. Cells were harvested at 72 hr postinfection. P450 contents were determined as described by Omura and Sato (21), and OR catalytic activity was determined by cytochrome *c* reduction as described in *Materials and Methods.*

0.04, 0.008, and 0.0016 pfu/cell at all combinations in 24-well plates. The resulting MOI ranges of v2A6 and vOR determined from coumarin 7-hydroxylase activity were subjected to further studies with

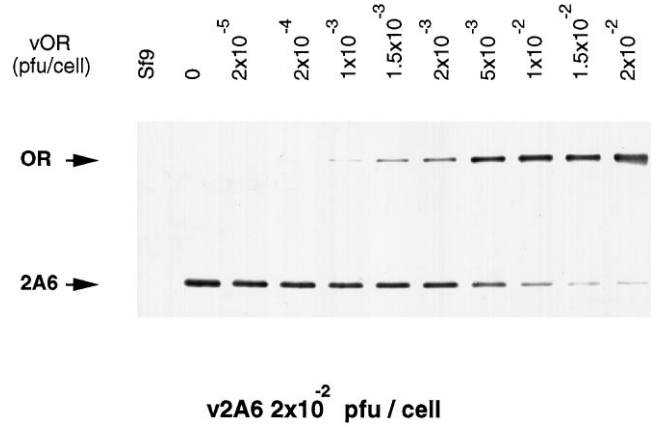

FIG. 3. *Immunoblot analysis of the effect of v2A6/vOR coinfection ratio on CYP2A6 total protein and OR protein expression level.*

One microgram of total cell lysate protein prepared for fig. 2 was subjected to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with an antibody mixture of 1:500 dilution of rabbit anti-rat 2A1 and 1:500 dilution of rabbit anti-rat OR, and detected by the alkaline phosphatase method.

smaller differences (1.5- to 2-fold) between each combination. The highest CYP2A6 activity for harvesting cells 72 hr postinfection was obtained by infection MOI of v2A6 of 4×10^{-1} with vOR of 4×10^{-2} pfu/cell (data not shown). This ratio, v2A6/vOR = 10/1, was extended to the spinner flask culture. MOI of v2A6 from 10^{-4} to 1.0 pfu/cell (vOR 10^{-5} to 10^{-1} pfu/cell) with 3- to 3.3-fold differences between infections were examined. The highest CYP2A6 activity was obtained with v2A6 infection MOI of 3×10^{-2} pfu/cell (vOR 3×10^{-3} pfu/cell) (fig. 1). Further studies examining the v2A6 MOI range from 1×10^{-2} to 1×10^{-1} with vOR 10-fold less, resulted in the highest CYP2A6 activity with v2A6 infection MOI of 1.5–2 $\times 10^{-2}$ pfu/cell (data not shown).

Coumarin 7-Hydroxylase Activity Was Dramatically Influenced by v2A6/vOR Infection Ratio. Sf9 cells (in stirred suspension culture) were coinfected with a fixed MOI of v2A6 (2×10^{-2} pfu/cell) and different MOI of vOR (0 and 2×10^{-5} to 2×10^{-2} pfu/cell). Coumarin 7-hydroxylase activity in the total cell lysate protein increased with increasing vOR MOI from 2×10^{-5} to 1×10^{-3} pfu/cell (or 1000- to 20-fold lower than v2A6 MOI, respectively; fig. 2*A*). A 50-fold increase in vOR resulted in a 5-fold increase in activity (and 7-fold over cells infected with v2A6 alone). Surprisingly, when the MOI of vOR was further increased 10-fold (from 1×10^{-3} to 1×10^{-2} pfu/cell, which was 50% of v2A6 MOI), the coumarin 7-hydroxylase activity decreased 14-fold to values below those obtained by infection with v2A6 alone. Immunoblot analysis (fig. 3) indicated that the OR protein was barely detectable in the cell lysate that produced the best catalytic activity (vOR of 10^{-3} pfu/cell); CYP2A6 total protein levels decreased with the increase of OR expression. The level of CYP2A6 holoenzyme and OR protein was also determined by CO-reduced difference spectra and cytochrome *c* reduction, respectively. The spectrally active P450 also decreased with the increased OR expression level (fig. 2*B*), whereas the maximum enzyme turnover number (4.56 nmol/min/nmol P450) was obtained when coinfecting Sf9 cells with vOR at MOI of 1×10^{-3} pfu/cell (fig. 2*C*).

Low MOI of v2A6 and vOR (v2A6/vOR = 10) Was Important for Obtaining High Enzyme Catalytic Activity. The requirement for a low MOI (substantially <1 pfu/cell) was surprising, and the dependence of activity on MOI was examined further. Increasing the MOI

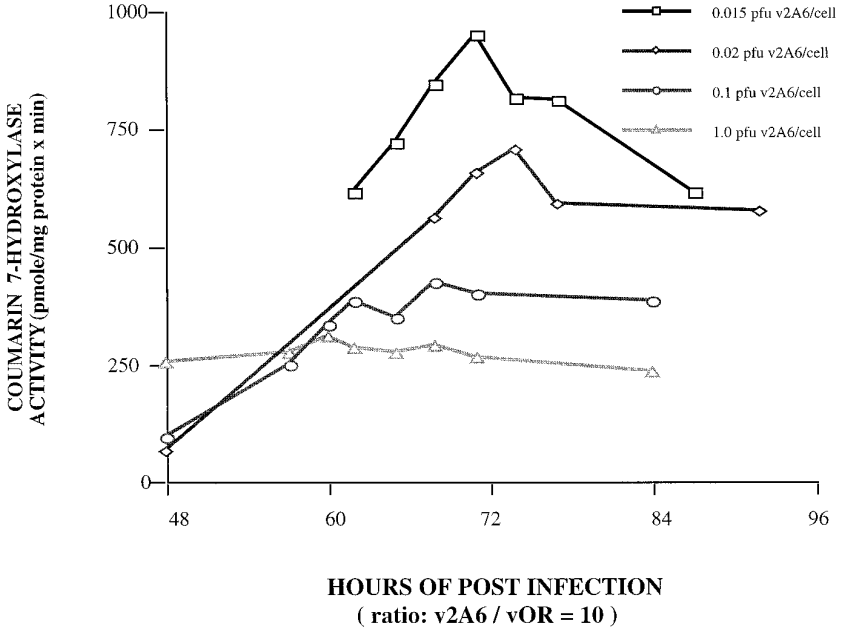

FIG. 4. *Time course of the effect of v2A6/vOR MOI (ratio 10) on coumarin 7-hydroxylase activity.*

Cells were coinfected with indicated v2A6 and 10-fold less of vOR, and harvested at the indicated time. Hemin was added. Coumarin-7-hydroxylase activity was determined as described in the legend to fig. 1.

of both v2A6 and vOR 5- to 50-fold did not improve maximum catalytic activity, but shifted the appearance of maximal catalytic activity from 72 hr postinfection to 60 hr postinfection (fig. 4). In contrast to the catalytic activity changes, which was decreased for infection with v2A6 at 2×10^{-2}, or no change in activity with v2A6 at 1×10^{-1} pfu/cell, both with vOR 10-fold less from 72 to 96 hr postinfection, immunoblot data (fig. 5) indicate both protein levels increased during this time for both infections. No increase in the amount of CYP2A6 or OR was seen when Sf9 cells were coinfected with v2A6 at 1.0 pfu/cell and vOR 10-fold less (fig. 5).

OR Protein May Decrease the P450 Content in Sf9 Cells. The spectral P450 content decreased in the interval from 72 to 96 hr postinfection when Sf9 cells were coinfected with v2A6 (2×10^{-2} pfu/cell) and vOR (1×10^{-3} pfu/cell) (fig. 6). When the same MOI or 10-fold higher MOI of wild-type baculovirus AcMNPV was substituted for vOR in coinfection, or Sf9 cells were infected with v2A6 alone, the spectral P450 contents all increased from 72 to 96 hr postinfection. In contrast, changes in CYP2A6 total protein levels by immunoblot analysis were essentially similar for all infections (fig. 7).

The Potential for Using Baculovirus for P450 Studies. cDNA-expressed CYP2A6 in the baculovirus system (total cell lysate) and human lymphoblastoid cells (microsomes) were compared (table 1). The spectral P450 content obtained in the insect cells coinfected with v2A6 and vOR was ~7-fold higher than the P450 content obtained in microsomes prepared from human lymphoblastoid cells transfected with an extrachromosomal vector containing CYP2A6 cDNA. However, coumarin 7-hydroxylase activity was only ~1.7-fold higher. Immunoblotting results (fig. 8) suggest that the expressed CYP2A6 total protein in the cell lysate was >7-fold higher than the protein detected in lymphoblast microsome. Interestingly, the OR protein expressed in baculovirus system possesses a higher molecular weight than OR protein expressed in the lymphoblasts, which has the same molecular weight as the OR protein detected in the human microsome (data not shown).

Discussion

In this study, we took advantage of the coumarin 7-hydroxylase activity assay (20), which can be performed in 24-well plates by fluorescent scanning, to determine the best infection MOI and ratio of v2A6 and vOR for coinfection. The best MOI, as determined by coumarin 7-hydroxylase activity, for coinfection in spinner flask, was ~20-fold less than coinfection in 24-well plates. Many conditions may account for this observation. For example, in the spinner flask, with more cell surface exposed to viruses (in 24-well plates, cells attach to plates), viruses produced by an initial round of infection may be able to infect more readily cells in the culture. Or, the shearing problem in the spinner flask can also increase the cell lysis, which was enhanced by nonoccluded virus infection (23). Therefore, a lower MOI for infection is required for spinner flask cultures. In early

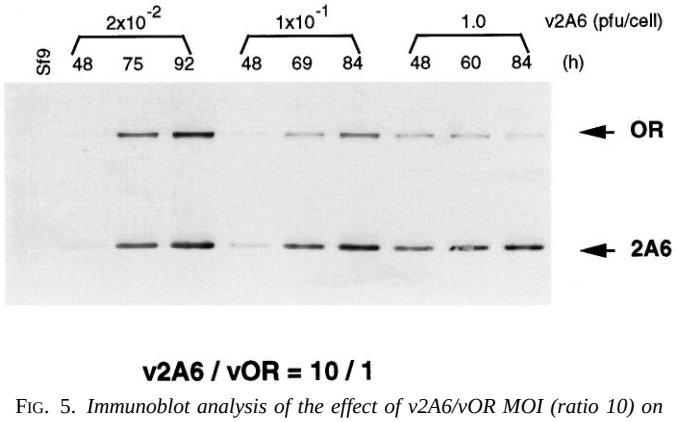

FIG. 5. *Immunoblot analysis of the effect of v2A6/vOR MOI (ratio 10) on the total CYP2A6 protein and OR protein level changes in the time course study.*

Cell lysates having the highest coumarin 7-hydroxylase activity in the time course study (fig. 4) were compared with the first and last point of collection. One microgram total cell lysate protein was loaded in each lane. Immunoblot was performed as described in the legend to fig. 3.

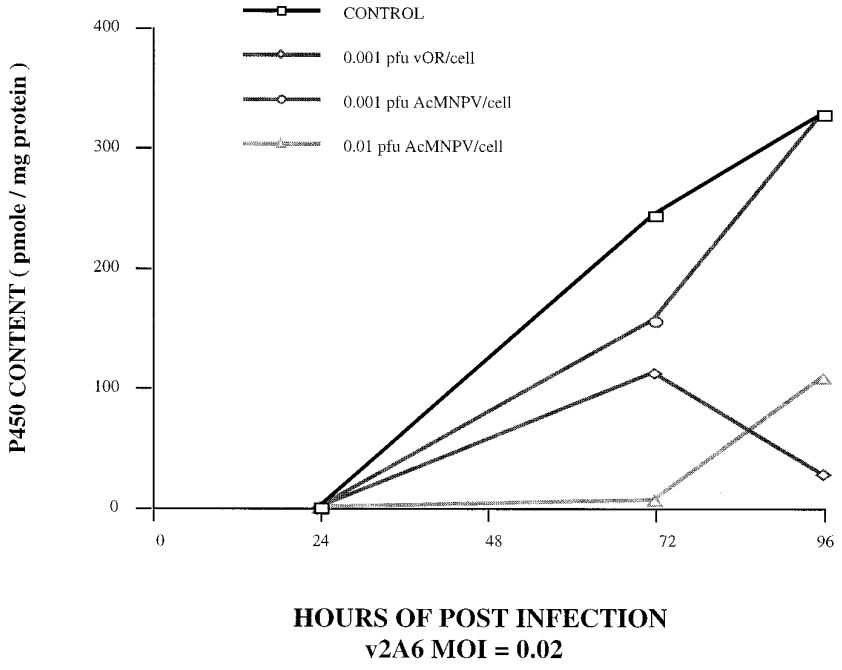

FIG. 6. *Coinfection of vOR with v2A6 decreases P450 content.*

Cells were infected with 2×10^{-2} pfu/cell of v2A6 alone (□), or coinfected with same amount of v2A6 and 1×10^{-3} pfu/cell of vOR (◇), or coinfected with 1×10^{-3} pfu/cell (○) or 1×10^{-2} pfu/cell (△) of wild-type baculovirus AcMNPV. Hemin was added 24 hr postinfection. Cells were harvested at 72 and 96 hr postinfection. P450 contents were determined as described in the legend to fig. 2.

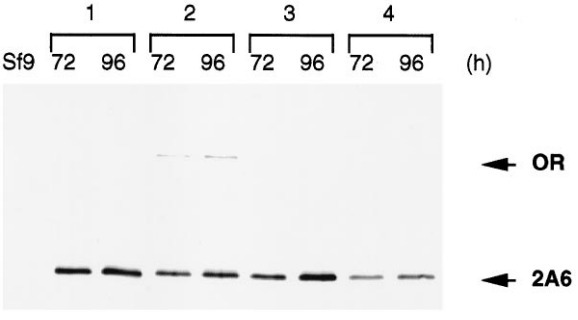

FIG. 7. *Immunoblot comparison of CYP2A6 total protein level between Sf9 cells coinfected with v2A6/vOR and v2A6/AcMNPV.*

One microgram total cell lysate prepared for fig. 6 was loaded for each lane. (*1*) Sf9 cells were infected with 2×10^{-2} pfu/cell of v2A6, or (*2*) coinfected with vOR at 1×10^{-3} pfu/cell, or (*3*) coinfected with wild-type baculovirus AcMNPV at 1×10^{-3} or (*4*) 1×10^{-2} pfu/cell. Immunoblot was performed as described in the legend to fig. 3.

reports, the MOI of P450 viruses was ~10 (24, 25). More recent reports have used lower MOI: a MOI of 4 in Petri dishes (26), a MOI of 1–2 (3, 5, 6), and an even lower MOI to 0.1 (4) in spinner flask.

In the present study, the best MOI of v2A6 as determined by coumarin 7-hydroxylase activity in spinner flask was $\sim 2 \times 10^{-2}$ pfu/cell when coinfecting with vOR at a MOI of 10- to 20-fold less. At this level of v2A6/vOR coinfection, the best coumarin 7-hydroxylase activity obtained in whole cell lysate (1300 pmol/min/mg total lysate protein) was comparable with or better than that observed in human liver microsomes (27). Therefore, the activity of the preparations should be adequate for many applications. Initial studies with human CYP2E1 indicate that similar properties (low overall MOI, v2E1 MOI at 2×10^{-2} pfu/cell and vOR at 1×10^{-2} pfu/cell at a vP450/vOR ratio of ~2) also apply to this protein. However, the optimal absolute conditions may be different. The different P450 and OR virus ratios required for optimal catalytic activities of CYP2A6 and CYP2E1 suggest that individual P450 expression level, P450 protein half-life, P450 turnover, and OR expression level may all influence the ratio for coinfection. The variation in the affinity of the OR protein to different P450s (28) may result in different P450s requiring different levels of OR for activity. The different distribution of charges at OR binding site(s) may cause different P450s to have different affinities to OR (29).

At this low level of initial infection seen in both studies, protein expression seems dependent on secondary or tertiary infection. Peak enzymatic catalytic activity could not be obtained by increasing initial infection MOI, thus suggesting that the best enzymatic catalytic activity depends on properly posttranslationally modified proteins accumulating in a right ratio as a result of primary, secondary, and possibly tertiary infection (30) of both viruses. These process cannot be obtained simply by increasing MOI for infection.

OR expression level was found to be important for coexpressed CYP2A6 protein catalytic activity. A clear optimum for vOR infection MOI was observed, and increasing vOR MOI above this level was associated with the destruction of the CYP2A6 holoenzyme and a net decrease in enzyme activity. OR degradation of the P450 holoenzyme may result from the OR-initiated oxidative damage of microsomal protein (31), whereas the deficiency of heme in cells coinfected with vOR is also suggested because the spectral P450 content decreased even when total CYP2A6 protein amount increased (compare figs. 6 and 7). OR has been shown to be involved in heme degradation by stimulating heme oxygenase (32) and oxidative heme destruction (33). Clearly, controlling OR expression level was important for achieving high enzyme catalytic activity. This problem could also be addressed by using different phase promoters, such as using late basic promoters (34–36) to express P450 and using very late promoters to express OR, or establishing stable cell lines (37) expressing a low to moderate level of OR. If this problem can be solved, it has the potential to

TABLE 1

Comparison of catalytic activity and P450 content between the baculovirus system and the human lymphoblastoid expression system

System	Fraction	Expressed Protein	Coumarin 7-Hydroxylase Activity		P450 Content	Reductase Activity
			pmol/min/mg	pmol/min/pmol		
					pmol/mg	*nmol/min/mg*
Insect	Cell lysate	CYP2A6	120	0.31	388	55
		OR	—	—	—	2849
		2A6/OR	1312	5.27	249	520
Lymphoblastoid	Microsomes	CYP2A6	770	22.0	35	29
		OR	—	—	—	3052

Insect cells were infected separately with v2A6 at a MOI of 0.02 or vOR at a MOI of 0.03. Insect cells coinfected at a MOI of v2A6/vOR of 0.015/0.001. Cells were harvested 72 hr postinfection, and lysates were assayed as described in *Materials and Methods*. Lymphoblastoid microsomes were isolated and assayed for activity as described previously (20).

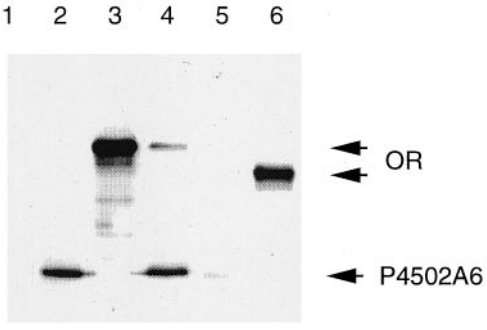

FIG. 8. *Immunoblot comparison of CYP2A6 total protein and OR protein expressed in baculovirus system and human lymphoblastoid cells.*

Lanes 1 to 4 are cell lysates from Sf9 cells (*lane 1*), Sf9 cells infected with 2×10^{-2} pfu/cell of v2A6 (*lane 2*), 3×10^{-2} pfu/cell of vOR (*lane 3*), or 2×10^{-2} pfu/cell of v2A6 and 1×10^{-3} pfu/cell of vOR (*lane 4*). *Lanes 5* and *6* are microsomes isolated from human lymphoblastoid cells transfected with an epichromosomal vector containing CYP2A6 cDNA (*lane 5*) or OR (*lane 6*). One microgram total protein was loaded for each lane, and the Western blot was performed as described in *Materials and Methods*.

increase dramatically the P450 catalytic activity in the baculovirus expression system and to simplify using the baculovirus system for P450 studies.

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