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ABOUT THE COVER:

Double immunofluorescence stain for dendritic marker MAP2 and neurofilament 200 in hippocampal neurons (see article on page 6)

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TEV PROTEASE, RECOMBINANT: A SITE-SPECIFIC PROTEASE FOR EFFICIENT CLEAVAGE OF AFFINITY TAGS FROM EXPRESSED PROTEINS

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Overproduction of proteins from cloned genes using expression vectors for *E. coli* and eukaryotic hosts has increased the quantity of protein produced. Recently, affinity purification using fusion proteins [*i.e.*, glutathione-S-transferase (GST), maltose binding domain (MBD), and polyhistidine sequences, cloned and expressed as a part of the protein of interest (1-3)] has allowed single column purifications of many proteins. Although these affinity tags facilitate purification of the protein, their presence may affect important characteristics or functions of the protein to be studied. Removal of the tag from the protein of interest can be accomplished with a site-specific protease such as Factor Xa, thrombin, or enterokinase (4).

The tobacco etch virus (TEV) protease is a new site-specific protease (5) that has a seven-amino-acid recognition site, **Glu-Asn-Leu-Tyr-Phe-Gln*Gly**. The glu, tyr, gln, and gly are required for efficient cleavage (6-8). Cleavage occurs between the gln and gly, resulting in a gly at the amino terminus of the protein of interest. TEV protease has been cloned and studied in the laboratories of Dr. W.G. Dougherty (Oregon State University) and Dr. S.A. Johnston (UT-Southwestern Medical

Center) (10). This recombinant form of TEV protease (rTEV) is expressed in *E. coli* and is purified via a polyhistidine tag. This tag also permits convenient separation of rTEV from the desired protein following cleavage of the fusion protein.

Experiments are presented showing the broad temperature range of rTEV and its activity on a variety of substrates. The high specificity, the high degree of purity, and the efficient cleavage at low temperature make rTEV an ideal choice for removal of tags from fusion proteins.

METHODS

The control substrate (9) for determining TEV protease activity is a 31-kDa protein consisting of the GST protein at the amino terminus, followed by the TEV cleavage site (TEV CS) and a 34-amino-acid carboxyl terminus. Cleavage of this GST.TEV CS.34 amino acid protein by TEV protease results in a 27-kDa protein. The standard assay (30 μ l) contained 3 μ g control substrate in 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 1 mM DTT, and varying amounts of TEV protease. The reaction was incubated at 30°C for 1 h, stopped by addition of 30 μ l 2X protein loading buffer, boiled for 5 min, and electrophoresed on a 10% GIBCO BRL SDS-Polyacrylamide Gel system (Cat. No. 15574). The percent substrate hydrolyzed was determined by scanning the gels after staining with GIBCO BRL BLUPRINT™ Fast-PAGE Stain (Cat. No. 15587). One unit of TEV protease is the amount of enzyme necessary to cleave \geq 95% control substrate (3 μ g) in 1 h at 30°C.

TEV Protease, Recombinant (Cat. No. 10127), is expressed in *E. coli* as a fusion protein to polyhistidine. The protein is purified on a Ni⁺⁺-NTA matrix (Qiagen). The enzyme is assayed for contaminating nonspecific protease activity by incubation with azocasein for 24 h. The presence of endonucleases, exonucleases, and RNase contamination is also assayed.

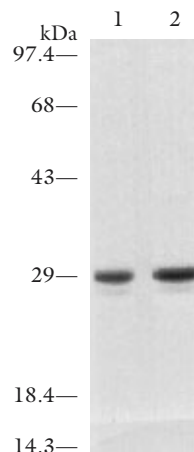


FIGURE 1. Purity of TEV Protease, Recombinant. Lane 1, 2 μ g, and lane 2, 4 μ g of rTEV.

The TEV cleavage site (TEV CS) was also cloned into the pGEX.3X vector (encodes GST) and the pMal.C2 vector (encodes MBD). The marker protein, bacterial alkaline phosphatase (BAP), was cloned downstream of the cleavage site in both vectors. The resulting proteins are GST.TEV CS.BAP (72 kDa) and MBD.TEV CS.BAP (85 kDa). BAP was also cloned into pMal.C2 and pGEX.3X without the TEV cleavage site. The resulting proteins contain the factor Xa cleavage site and are named GST.Xa CS.BAP and MBD.Xa CS.BAP, respectively.

The cleavage site can be introduced into expression vectors by synthesizing a ds DNA oligonucleotide containing the nucleotides that encode the recognition site amino acids. The recognition site can be cloned directly adjacent to the GST domain (pGEX vectors) or to MBD (pMal vectors). To improve efficiency of rTEV cleavage for HIS tag vectors, the addition of a spacer arm sequence such as Asp-Tyr-Asp-Ile-Pro-Thr-Thr inserted at the carboxyl terminus of the HIS tag, upstream of the rTEV cleavage site, is recommended.

RESULTS AND DISCUSSION

rTEV has an apparent molecular weight of 29 kDa when purified via the polyhistidine tag.

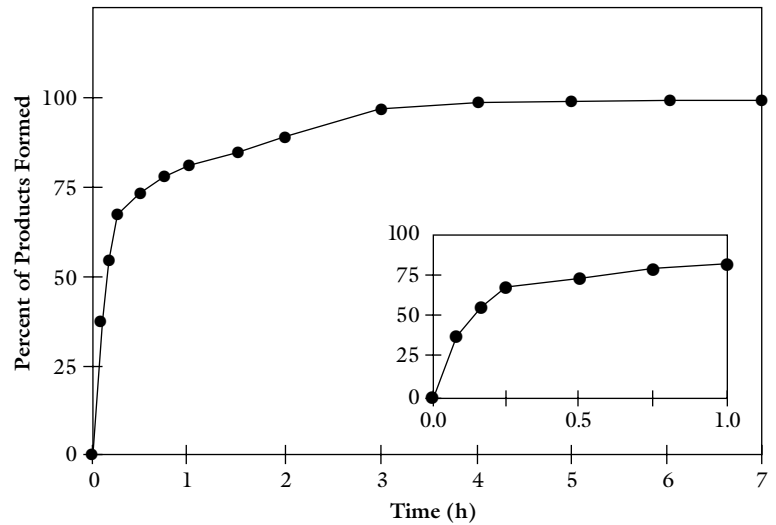


FIGURE 2. Time course of the cleavage reaction. Control substrate (3 μ g) was incubated with 0.5 units of rTEV at 30°C for varying times. The insert is an expanded view of the first hour of digestion.

The protein had >95% single band purity (figure 1). There was no detectable nonspecific protease, endonuclease, exonuclease, or RNase contamination (data not shown).

The affect of a variety of potential protease inhibitors on the cleavage of rTEV at 30°C was examined. rTEV was not inhibited by 2.5% sucrose or 0.01% TRITON X-100. However, SDS at 0.01% completely inhibited rTEV

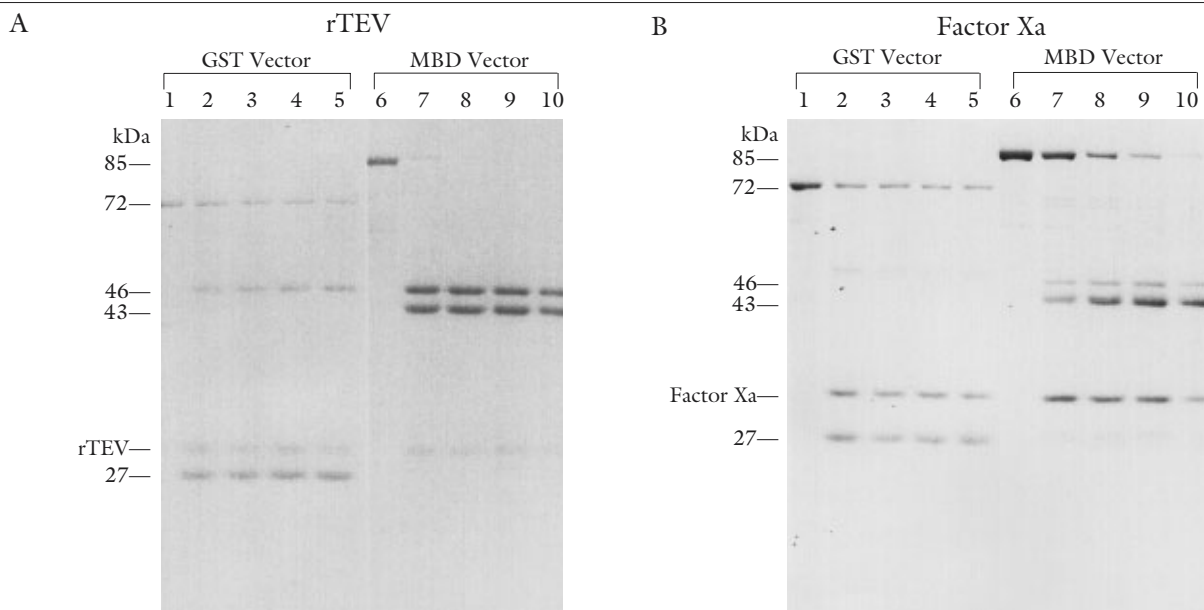


FIGURE 3. Comparison of rTEV and Factor Xa. Panel A. GST.TEV CS.BAP and MBD.TEV CS.BAP were incubated in the absence of rTEV for 24 h (lanes 1 and 6, respectively) or in the presence of 10 units (1 μ l) rTEV for 1, 2, 3, 5 h (lanes 2-5 and 7-10) at 20°C. Panel B. GST.Xa CS.BAP and MBD.Xa CS.BAP were incubated in the absence of Factor Xa (New England Biolabs) for 24 h (lanes 1 and 6, respectively) or in the presence of 1 μ g (1 μ l) Factor Xa for 1, 2, 3, 5 h (lanes 2-5 and 7-10) at 20°C. Uncleaved MBD.BAP, 85 kDa; uncleaved GST.BAP, 72 kDa; BAP, 46 kDa; MBD CS, ~43 kDa; and GST CS, 27 kDa.

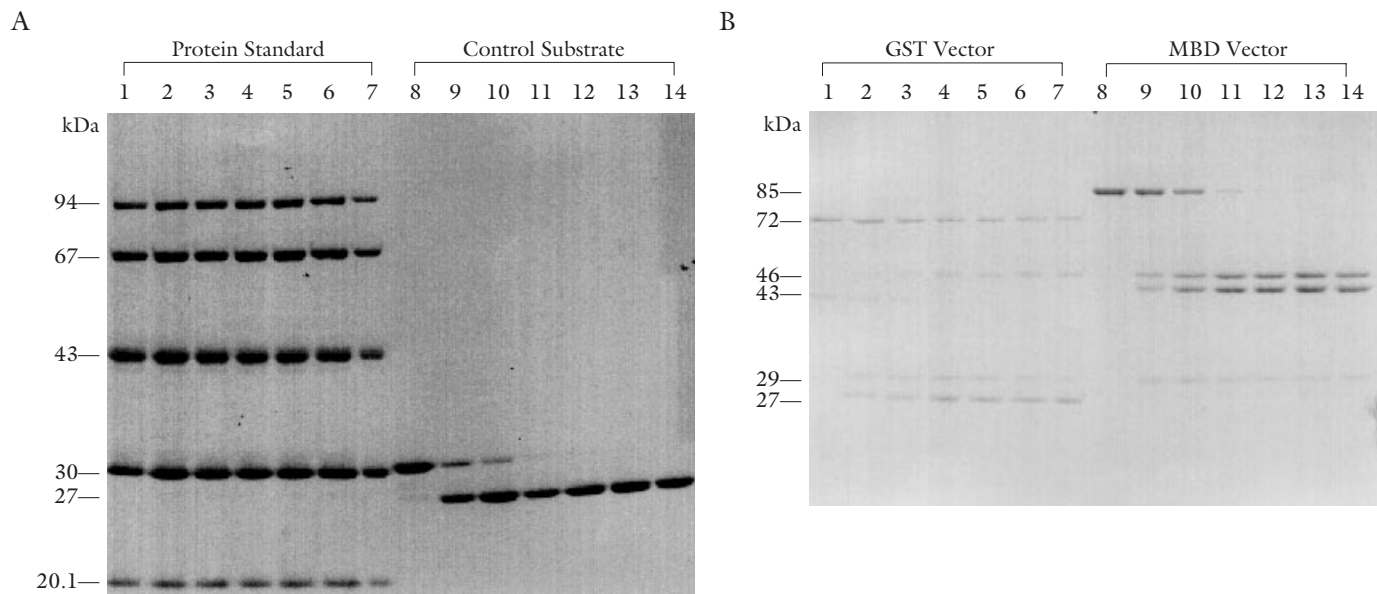


FIGURE 4. Cleavage of various proteins with rTEV at 4°C. The substrates (3 µg) were incubated at 4°C without or with rTEV protease for 1, 2, 4, 6, 9, or 24 h. Panel A. Protein standards were incubated in the absence (lane 1) or presence of 20 units of rTEV (lanes 2-7). Control substrate was incubated in the absence (lane 8) or presence of 2 units of rTEV (lanes 9-14). Panel B. GST.TEV CS.BAP was incubated in the absence (lane 1) or presence of 10 units rTEV (lanes 2-7). MBD.TEV CS.BAP was incubated in the absence (lane 8) or presence of 10 units rTEV (lanes 9-14). Uncleaved GST.TEV CS.34, 31 kDa; uncleaved MBD.BAP, 85 kDa; uncleaved GST.BAP, 72 kDa; BAP, 46 kDa; MBD CS, ~43 kDa; rTEV, 29 kDa; and GST CS, 27 kDa.

activity. Control substrate was completely cleaved in the presence of 0.05, 0.1, and 0.5 M urea. In the presence of 1 M urea, ~50% of the control substrate was cleaved. Also, the enzyme is not inhibited by PMSF, aprotinin, or leupeptin (5); therefore, fusion proteins may be purified in the presence of these inhibitors.

The unit activity of rTEV was determined with a control substrate, GST.TEV CS.34. A titration of rTEV resulted in a typical hyperbolic curve representative of a single substrate reaction (data not shown). The initial velocity of the reaction was linear, representative of an enzyme obeying steady state kinetics (figure 2 insert). The unit definition is defined as the amount of rTEV necessary to cleave 3 µg of substrate to 95% completion. This unit reflects

the need to provide a nominal amount of enzyme required to obtain near quantitative removal of the fusion protein.

The rTEV protease was compared to Factor Xa protease (figure 3). Reactions were performed at 20°C (recommended temperature for Factor Xa). For the GST fusion proteins, complete cleavage (with either the TEV or Factor Xa cleavage site) results in the appearance of two protein bands (46 kDa and 27 kDa). Cleavage of GST.TEV CS.BAP with rTEV (10 pmol enzyme) was ~75% complete in 5 h, whereas cleavage of GST.Xa CS.BAP with the Factor Xa (28 pmol) was only 50% in 5 h. For the MBD fusion proteins, complete cleavage results in the appearance of two protein bands (46 kDa and 43 kDa). Complete cleavage of the rTEV cleavage site was achieved in 2 h. In contrast, cleavage was not complete after 5 h with Factor Xa. These results demonstrated that cleavage is substrate dependent for both rTEV and Factor Xa. In addition, significantly more cleavage was observed with rTEV than Factor Xa even though greater than twice as many moles of Factor Xa were used.

rTEV protease was active over a broad temperature range (table 1). Rapid cleavage was observed at 30°C, with no significant difference

TABLE 1. Cleavage with rTEV protease at various temperatures.

Time (h)	Percent Product Produced			
	4°C	16°C	21°C	30°C
0.5	25	55	57	75
1	48	80	78	95
2	74	99	99	99
3	84	99	99	99

Control substrate (3 µg) was incubated with 1 unit rTEV protease.

in cleavage being observed at 37°C (data not shown). Approximately 80% cleavage was observed in 1 h at both 21°C and 16°C. Additionally, approximately 50% cleavage was observed at 4°C.

Fusion protein cleavage at 4°C by rTEV was examined with three substrates, GST.TEV CS.BAP, MBD.TEV CS.BAP, and a protein standard (figure 4). The protein standard was tested to determine if there was any change in specificity at the reduced temperature. No change in the protein standard was observed after incubation with 20 units of rTEV for 24 h whereas 2 units of rTEV resulted in complete cleavage of the control substrate in 4 h. rTEV maintained its ability to cleave specifically at the recognition site at 4°C.

Hydrolysis of GST.TEV CS.BAP at 4°C was less efficient, requiring more than 24 h for 75% cleavage (figure 4, panel B). In contrast, the MBD.TEV CS.BAP protein was hydrolyzed to completion in <6 h at 4°C. These results show that the activity of rTEV is also substrate dependent at 4°C.

These experiments show a difference in cleavage with rTEV and Factor Xa for GST and MBD affinity tags. Other factors may lead to a difference in cleavage, so each fusion protein should be tested to determine the optimal concentration and reaction temperature for rTEV.

TEV Protease, Recombinant, is a highly specific protease that cleaves a variety of fusion proteins. The efficiency of cleavage is depen-

dent on both the tag and the protein fused to the carboxyl terminus of the TEV cleavage site. rTEV can efficiently recognize its cleavage site at 4°C. We recommend that cleavage be performed at 4°C for any heat labile protein.

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EDITOR'S NOTE

A prokaryotic expression vector containing a TEV cleavage site and a polyhistidine affinity tag is available for field tests. Contact Dr. Deborah Polayes at Life Technologies.

NEUROBASAL™ MEDIUM/B27 SUPPLEMENT: A NEW SERUM-FREE MEDIUM COMBINATION FOR SURVIVAL OF NEURONS

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Growth of neurons occurs by process outgrowth. Many neurons die during this differentiation. The surviving neurons maintain unfragmented processes. B27 was developed as a supplement to D-MEM for the growth of hippocampal neurons by optimization of over 20 components of a previously published serum-free supplement called B18 (1). With rat embryonic hippocampal neurons, D-MEM with B27 produced a 4-day neuron survival above 50%, independent of plating density above 160 plated cells/mm² (2). However, the combination of B27 with D-MEM or D-MEM:F12 for neuron survival has several problems including, rapid deterioration of the ability of D-MEM:F12 to support neuron survival and the presence of potential excitotoxic amino acids in both basal media. Basal media (including D-MEM and D-MEM:F12) were developed for rapid cell division of somatic cells, so it is not surprising that these media are not optimal for the survival and differentiation of neurons.

Since freshly made D-MEM:F12 produced higher neuron survival than D-MEM in our studies, Neurobasal medium was developed by optimizing concentrations of the components found in D-MEM:F12 but absent from D-MEM: alanine, asparagine, cysteine, glutamate, proline, and vitamin B12 (2). In addition, osmolality, glutamine, and sodium bicarbonate concentrations were optimized. Neurobasal medium, therefore, is a modified D-MEM:F12 with a lower concentration of several amino acids. In addition, ferrous sulfate and the excitatory amino acids glutamate and aspartate were eliminated. In Neurobasal medium with B27, excellent long-term survival was achieved after 4 weeks in culture with greater than 90% viability for hippocampal neurons plated at 640 cells/mm² and greater than 50% viability for cells plated at 160/mm² (2). This paper shows the improved performance of Neurobasal medium with B27 over D-MEM with either B18 supplement, serum, or N2 supplement (3). Also, the stability of B27 supple-

ment, the need for glutamate in the medium, and maintenance of immunoreactivity for two neuronal markers neurofilament and MAP2 in Neurobasal/B27 are examined.

METHODS

Isolation and plating of rat hippocampal neurons. All salt solutions, media, and reagents were from Life Technologies. Embryos were recovered by c-section under nembutal anesthetic. Individual cells were isolated by trituration 10 times in 1 ml of Hanks' Balanced Salt Solution (HBSS) without Ca⁺⁺ and Mg⁺⁺ and supplemented with 1.0 mM sodium pyruvate and 10 mM HEPES (pH 7.4) using a 9-inch siliconized pasteur pipet with the tip barely fire polished. Divalent cations were restored by dilution with 2 volumes HBSS with Ca⁺⁺ and Mg⁺⁺ supplemented as above. After allowing nondispersed tissues to settle for 3 min, the supernate was transferred to a 15-ml tube and centrifuged for 1 min at 200 × g. The pellet was gently resuspended in 1 ml HBSS per brain and an aliquot added to trypan blue stain for a haemocytometer count. The culture vessels were coated with a 0.05-mg/ml solution (0.15 ml/cm² surface area) of cold poly-D-lysine (MW 30,000 – 70,000) and incubated for 1 h or overnight. The poly-D-lysine solution was stored at -20°C in polystyrene tubes and was prescreened for toxicity.

Vessels were washed with sterile, deionized cell culture grade water. Vessels can be stored for up to 2 weeks at 4°C to 10°C in sterile deionized, distilled water. If vessels are stored, remove water ~1 h prior to use. To Neurobasal medium (Cat. No. 21103), add 0.5 mM L-glutamine, 25 µM glutamate, and B27 supplement [2 ml of B27 50X concentrate (Cat. No. 17504) to 100 ml Neurobasal medium]. Cells were seeded at the desired densities. Cultures maintained longer than 4 days should have half the medium changed to Neurobasal/B27 without glutamate on day 4 and then once per week. If the initial culture density is higher than 640 cell/mm², the medium should be changed twice a week.

Immunofluorescence staining. Cells were plated at 400 cells/mm² and grown for 5 days in Neurobasal/B27. Neurons were fixed for 30 min in 3.7% formaldehyde in PBS; and rinsed and blocked in 1% BSA, 1% normal goat serum, 0.05% TRITON[®] X-100. Primary antibodies were added together and incubated overnight with the neurons at 4°C. Rabbit anti-neurofilament 200 (1:50) was detected with rhodamine-conjugated goat anti-rabbit IgG (1:500). Mouse anti-MAP2 (1:200) was detected with fluorescein-conjugated goat anti-mouse IgG (1:100).

RESULTS AND DISCUSSION

A discriminating measure of the ability of culture medium to maintain cell viability is to measure survival at low cell plating densities for periods longer than 3 days. For neurons, this is particularly true due to supposed needs for trophic factors. After 1 day in culture, survival correlated well with the number of cells with processes (data not shown). After 4 days in culture, survival was much more discriminating for the effect of medium components. The 5 day survival of neurons in Neurobasal/B27 was far better than cells cultured in D-MEM with B18 and Neurobasal/N2 at all plating densities (figure 1). At all but the highest plating density, Neurobasal/B27 was superior to Neurobasal medium with 5% FBS.

To examine the stability of B27, neurons were plated at 160 cells/mm² in NEUROBASAL medium with freshly thawed B27 or B27 stored at 4°C for 2 months. Four-day survival values were 60% and 64%, respectively. There appears to be no significant loss of activity at 4°C over a period of 2 months. Also, B27 stored at -20°C for 1 year has not shown any loss in neuron survival.

One of the advantages of Neurobasal medium is the omission of the excitatory transmitter amino acids, glutamate and aspartate. Glutamate excitotoxicity is not only a subject of great experimental interest with relevance to hypoxia-ischemia, hypoglycemia, and epilepsy (4), but could be relevant to the ability to maintain neurons in culture. During embryogenesis, the brain capillary endothelium or blood-brain barrier is not as tightly sealed as it is postnatally. Therefore, differentiating neurons with needs for neurite growth may require

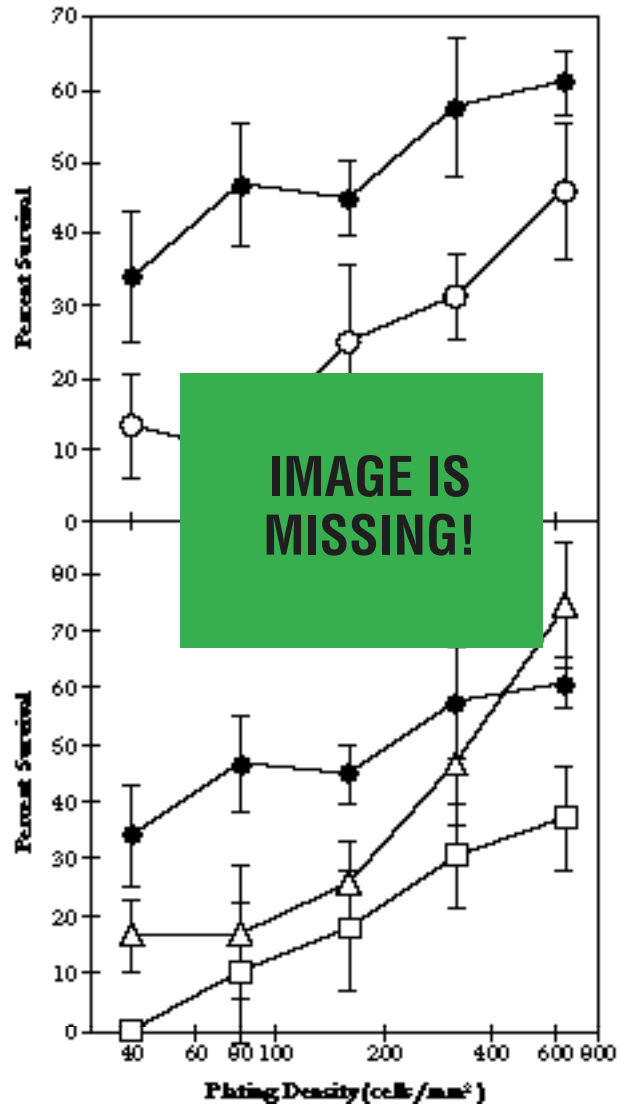


FIGURE 1. Comparison of Neurobasal/B27 to D-MEM and Neurobasal with other supplements. Cells isolated in HBSS from the hippocampus of 18-day-gestation rat embryos were plated at the indicated densities in Neurobasal/B27 (●), D-MEM/B18 (○), Neurobasal/N2 (□), and Neurobasal/5% FBS (▼). After 5 days of growth, live and dead neurons were counted using fluorescein diacetate and propidium iodide as described (2). Survival is the ratio of live to total neurons.

glutamate at a concentration closer to that found in serum. Adult rat serum glutamate levels are around 11 µg/ml. With serum-free medium, it was possible to optimize the glutamate concentration for survival of embryonic hippocampal neurons. Survival after 4 days indicated an optimum near 25 µM (3.7 µg/ml). Figure 2 examines the effect of the continued presence of glutamate. Neurons originally plated in 25 µM glutamate were either left to grow, or one-third of the medium was changed to fresh medium (D-MEM/B27) with or with-

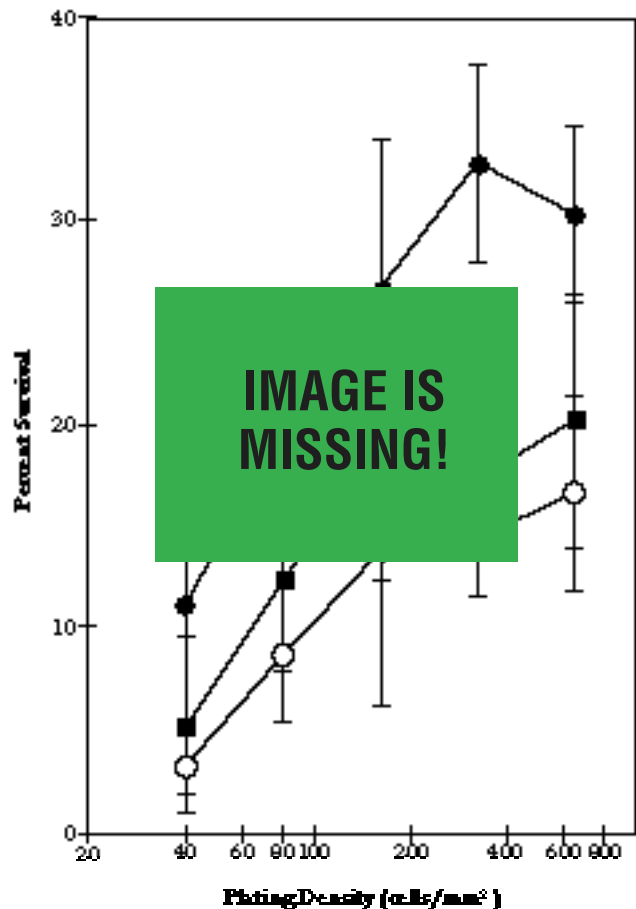


FIGURE 2. The long-term effect of glutamate on neuron survival. Embryonic hippocampal neurons were plated in D-MEM with B27 at the indicated densities. After 4 days of growth, medium was either not changed (○), or one-third was replaced with fresh medium with (■) or without (●) 25 μ M glutamate. Survival was determined on day 8.

out 25 μ M glutamate after 4 days. Only one-third of the medium was changed to minimize changes in trophic factors present in the conditioned medium. At 320 cells/mm², survival that was 60% on day 4 drops to 14% on day 8 without changing the medium. Changing one-third of the medium on day 4 to medium without glutamate increased survival to 33%. The increase in survival was due to fresh medium without glutamate since the survival was not improved by a medium change with glutamate (17% survival). Subsequent tests with Neurobasal/B27 have produced better long-term survivals by replacing one-half of the medium with glutamate-free medium on day 4 (data not shown). Further medium changes depend on cell density: above 160 cells/mm², change medium every 3 to 4 days and below 160 cells/mm², change the medium once a week.

One- to three-day cultures are characterized by several dendritic processes and a single axonal process (5). Culturing cells for longer periods leads to a more dense network of dendritic processes and continued growth of axons. The cover photograph shows the characteristic features of neuronal cells grown in Neurobasal/B27 for 5 days. Dendritic processes have tapering arbors and frequent branches at acute angles. Axons are identified by their small, uniform caliber and branching at right angles. Dendrites, but not axons, were stained with the microtubule-associated protein MAP2 (yellow/green) (6). Anti-neurofilament was used to stain axons (red), although this cytoskeletal component is not exclusively an axonal marker.

Neurobasal medium with B27 also supported the growth of neurons from embryonic rat striatum, substantia nigra, septum and cortex for 1 week (table 1), as well as dentate gyrus and cerebellum from neonatal rats (Brewer, manuscript in preparation). Even though levels of survival were adequate, further studies are needed to optimize the B27 supplement for each cell type. Recent studies have also shown that adult rat hippocampal neurons can be isolated grown in Neurobasal/B27 (Brewer, manuscript in preparation). This serum-free medium combination was shown to be effective for the growth of tumor cell lines of neuronal origin (B104, PC12). Therefore, support for other CNS primary neurons and neuroblastomas is likely.

In serum-supplemented media, glial cells continue to proliferate which usually necessitates the addition of cytotoxic inhibitors (7) to

TABLE 1. Survival of other rat CNS neurons in Neurobasal/B27.

Cell Type	Age ^a	Survival Relative to Hippocampal Neurons
Cortex	E18	82%
Septum	E18	59%
Substantia nigra	E18	47%
Striatum	E18	66%
Cerebellum (granule cells)	P8	91%
Dentate gyrus	P4	66%

Cells were plated at 320/mm² and grown for 4 days. Relative survival was calculated by dividing survival for cell type by survival for E18 hippocampal neurons.

^aE = Embryonic age gestation, P = Postnatal age in days.

the medium to prevent glial cell overgrowth. In Neurobasal/B27, glial growth is reduced to less than 0.5% resulting in a nearly pure population of neurons (2).

Neurobasal/B27 will be useful in studies of neuronal development, plasticity, electrophysiology, gene expression, pharmacology, and neurotoxicity. As with other serum-free media, Neurobasal/B27 should also be useful in studies of growth factors, hormones, cytokines, and other bioactive compounds. In addition, Neurobasal/B27 allows the study of individual isolated neurons for several weeks without a glial cell feeder layer.

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GROWTH AND METABOLISM OF A MURINE HYBRIDOMA IN CULTURES CONTAINING GLUTAMINE-BASED DIPEPTIDES

ABSTRACT

A murine hybridoma was grown in media containing glutamine, alanyl-glutamine (ala-gln), or glycyl-glutamine (gly-gln) as a major carbon source. Cell yields were slightly higher in the presence of either dipeptide and with equivalent antibody productivity. A higher concentration of gly-gln (20 mM) was required to obtain comparable cell yields to the ala-gln (6 mM) or gln (6 mM) based cultures. A noticeable lag phase in the dipeptide cultures was attributed to a period of dipeptide hydrolysis catalyzed by an enzyme with higher specificity for ala-gln than gly-gln. The peptidase enzyme was present in the cytosolic fraction of the cell.

Glutamine is included in most culture media formulations used for the growth of mammalian cells at concentrations up to 10 times higher than other amino acids due to its importance as a precursor for nucleic acids and proteins as well as its role as an energy source (1,2). However, glutamine is chemically unstable with significant

degradation rates even when stored at 4°C (3). This severely limits the shelf life of liquid media and most certainly prohibits the use of autoclaving as a means of sterilization. A further problem with the use of glutamine is that both its chemical breakdown and cellular metabolism lead to the formation of ammonia, which can be inhibitory to cell growth, a particular problem for the culture of certain cell lines (4).

For these reasons, attempts have been made to replace glutamine by non-ammoniogenic analogues. These are required to be chemically stable compounds that can act as alternative growth substrates. Glutamate can be an effective substitute if the cells are adapted (5), but in some cases the rate of membrane transport may be too low to allow sufficient cellular uptake (6). Several recent reports have focused on the replacement of glutamine by glutamine-containing dipeptides such as alanyl-glutamine (ala-gln) or glycyl-glutamine (gly-gln) (7-9).

The dipeptides are autoclavable (figure 1) and this increased stability compared to glutamine is likely to confer an extended shelf life to dipeptide-based media. Furthermore, they

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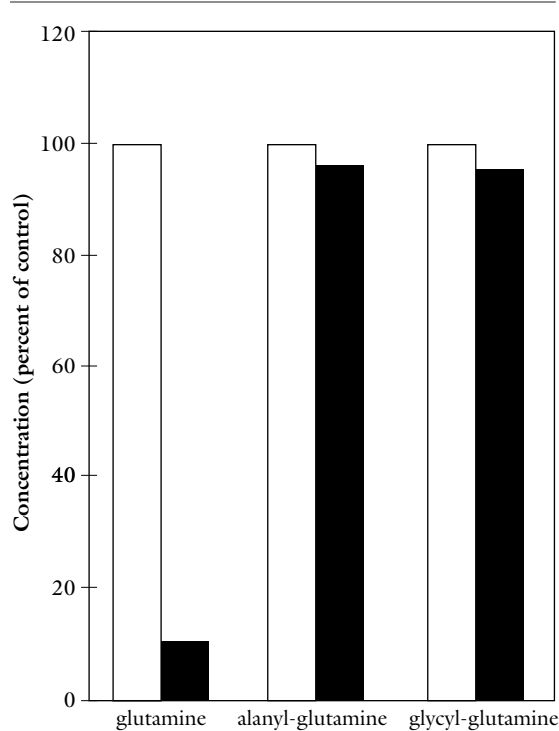


FIGURE 1. Effect of autoclaving on gln and dipeptide stability. Samples of culture medium containing 6 mM gln, 6 mM ala-gln, or 6 mM gly-gln were autoclaved at 120°C and 15 psi for 20 min. Concentration before autoclaving (□), after autoclaving (■).

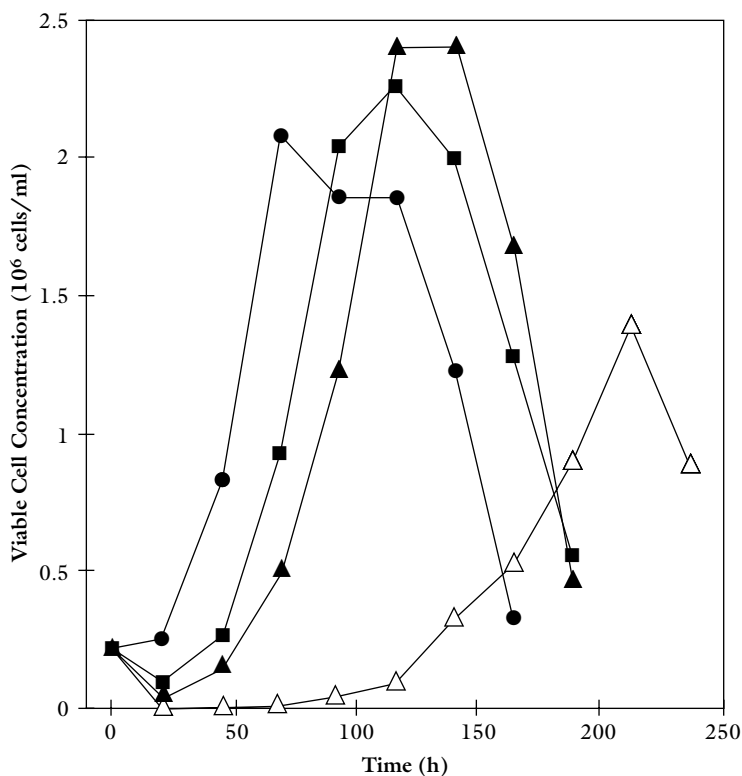


FIGURE 2. Cell growth in media supplemented with gln or dipeptides. Medium was supplemented with 6 mM gln (●), 6 mM ala-gln (■), 6 mM gly-gln (△), or 20 mM gly-gln (▲).

have the potential for improved cell yields in batch culture because they are less ammonia-genic. In this report, a mouse hybridoma, CC9C10, was grown in media containing ala-gln or gly-gln. Growth characteristics, antibody production, and cellular metabolism of the hybridoma were compared. We also report on some of the characteristics of the peptidase enzyme that is necessary for the hydrolysis and metabolism of the dipeptides.

MATERIALS AND METHODS

Cell culture. The murine hybridoma, CC9C10, was obtained from the American Type Culture Collection. The cells produce a monoclonal antibody (IgG₁) specific for insulin. Cells were grown in 25 ml GIBCO BRL D-MEM supplemented with 10% FETALCLONE® (Hyclone) and either 6 mM gln, 6 mM ala-gln, 6 mM gly-gln, or 20 mM gly-gln. In order to allow adaptation to the specific substrates, the cells were grown for 6 passages (24 generations) in each medium prior to the experimental work described here. Cells were counted by the trypan blue exclusion method using a Neubauer haemocytometer.

Media components. Amino acid concentrations were determined by HPLC separation of OPA derivatives. Ammonia was determined with a specific electrode (Orion) and glucose and lactate with a specific analyzer (YSI).

Antibody production. Antibody concentrations were determined with a PROANA™ Mabs column (Hyclone).

Peptidase activity. Lysates were prepared (10) from cells taken from mid-exponential phase of a culture, and protein content was determined by the bicinchoninic acid assay. Peptidase assays were conducted by incubating 0.05 µg/ml protein with 6 mM dipeptide (ala-gln, gly-gln, gly-glu, glu-trp, or gly-D-phe) in D-PBS containing 50 mM HEPES buffer (pH 7.4) at 37°C for 1 h. Free amino acids were quantified by HPLC as a means of determining hydrolytic activity.

Cellular fractionation. Cells were lysed in 5 mM Tris-HCl (pH 7.4), in ~1 min by 12 passes of a cooled Potter-Elvehjem homogenizer. Three stages of centrifugation at 3,000 × g for 10 min, 31,000 × g for 90 min, and 100,000 × g for 90 min led to 3 pellet samples (P1 to P3) and 3 supernatant samples (S1 to

S3). All pellets were washed in PBS between centrifugation steps.

RESULTS AND DISCUSSION

Cell growth and productivity. Figure 2 shows the growth characteristics of the cells in media containing gln or dipeptides. High yields were obtained from 3 media with a maximum cell density obtained in 20 mM gly-gln. Cell growth and yield with 6 mM gly-gln was poor and was not considered for further analysis. A decrease in viable cell concentration occurred in the first 24 h in the gly-gln (20 mM) and ala-gln cultures. These growth characteristics have been consistent in our laboratory for up to 40 culture passages. The slight increase of the lag phase in the dipeptide cultures is attributed to a period of extracellular release of a peptidase and subsequent dipeptide hydrolysis. This allows a gradual increase in availability of glutamine to the cells. The length of the lag phase could be decreased by the addition of a minimal quantity of glutamine to the dipeptide cultures at inoculation. Antibody production was not significantly different in the gln, ala-gln, or gly-gln cultures, reaching maximum concentrations of ~200 $\mu\text{g}/\text{ml}$ and specific production rates during the exponential phase of 1.0 to 1.3 $\mu\text{g}/10^6$ cells/h in each (figure 3).

Substrate utilization and by-product formation. Analysis of the glutamine level of 3 cultures is shown in figure 4. In D-MEM with gln, the gln was depleted in 116 h with a concomitant increase in ammonia concentration to 4.4 mM. This is a typical pattern of substrate utilization associated with hybridoma cell growth (1,2). In D-MEM with ala-gln, the gln concentration increased from zero to 4.2 mM in the first 24 h. This was coincident with the lag phase and is likely to be associated with a period of dipeptide hydrolysis. The increase in gln content of the D-MEM with gly-gln cultures over this period was considerably lower despite the higher content of the dipeptide in the medium. This suggests an enzymatic breakdown of dipeptides, but with greater specificity for ala-gln than gly-gln. The increase in gln concentration during the decline phase of D-MEM with gly-gln may be explained by dipeptide hydrolysis, but in the absence of cellular gln consumption. The final ammonia concentration in the D-MEM with gly-gln

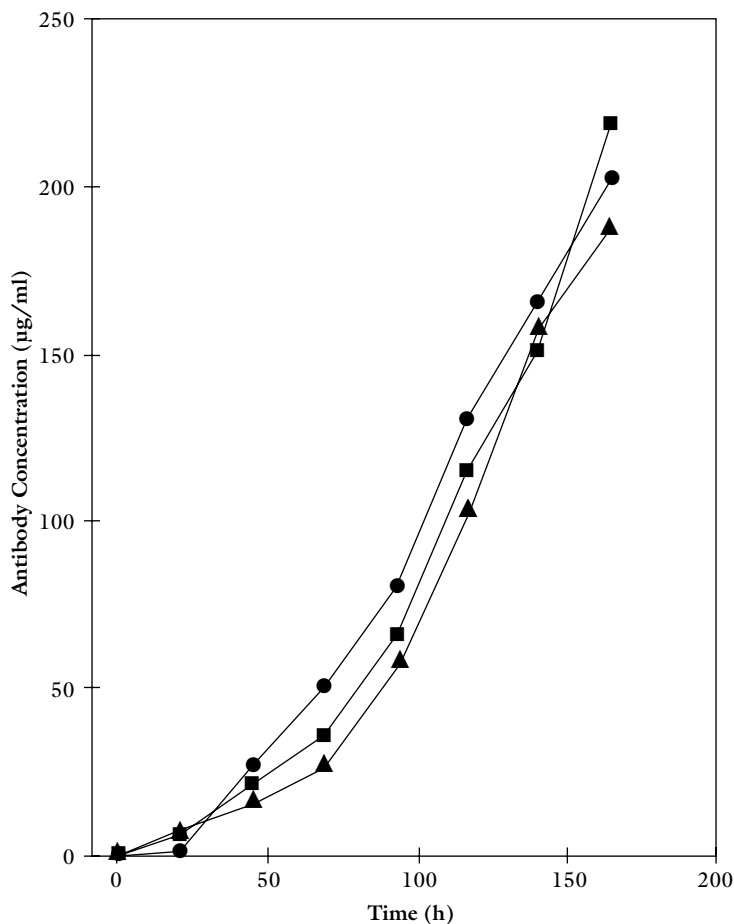


FIGURE 3. Antibody production. Cells were cultured in D-MEM containing 6 mM gln (●), 6 mM ala-gln (■), or 20 mM gly-gln (▲).

culture was significantly lower than in the other two cultures and may be explained by a lower gln consumption.

During the exponential phase, the specific glucose consumption in the D-MEM with gly-gln culture of 176 $\text{nmol}/10^6$ cells/h was nearly 50% lower than in the D-MEM with gln culture, and specific lactate production of 374 $\text{nmol}/10^6$ cells/h was almost 40% lower (figure 5). This may be explained by the need to sequester the increased intracellular ammonia by glycolytic intermediates as previously observed (5).

Specificity of peptidase activity. The specificity of intracellular peptidase activity was investigated by incubating a cell lysate with various dipeptides. Of 5 dipeptides tested, those containing glutamine showed the highest activity (table 1). The rate of hydrolysis of ala-gln was nearly twice that of gly-gln. This relatively higher specificity of peptidase for ala-gln indicates

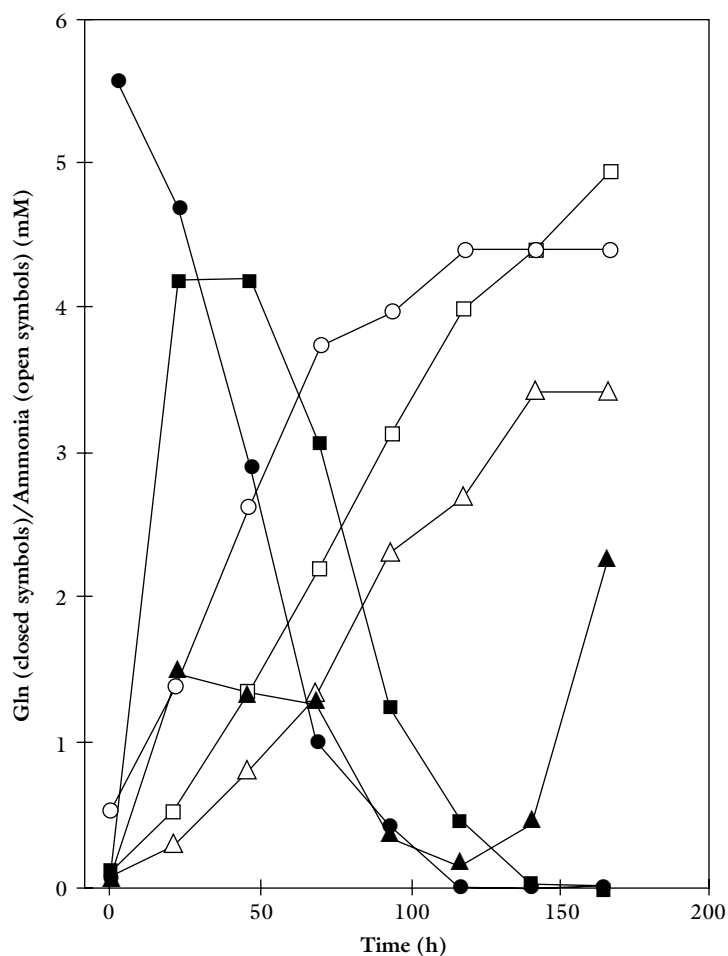


FIGURE 4. Gln and ammonia concentrations in culture. Cells were cultured in D-MEM containing 6 mM gln (●, ○), 6 mM ala-gln (■, □), or 20 mM gly-gln (▲, △).

why a correspondingly lower concentration of this dipeptide can be used to maintain cell growth comparable to D-MEM with gly-gln.

Cellular location of peptidase activity. The cell lysate was fractionated by differential centrifugation to pellet fractions, P1 (nuclei and plasma membrane sheets), P2 (smaller organelles and plasma membrane fragments), and P3 (all other noncytosolic material).

TABLE 1. Peptidase activity in CC9C10 cell lysate.

Dipeptide	Specific Rate of Hydrolysis (μmol dipeptide hydrolyzed/mg protein per min)
ala-gln	0.741
gly-gln	0.382
gly-glu	0.067
glu-trp	0.221
gly-D-phe	<0.004

Data are the average of 2 determinations.

TABLE 2. Peptidase activity in cellular fractions of CC9C10 isolated by differential centrifugation.

Fraction	Specific Activity ($\mu\text{mol}/\text{mg}$ protein/min)	
	ala-gln	gly-gln
P1	0.019	0.009
P2	0.108	0.050
P3	<0.004	<0.004
S1	0.415	0.190
S2	0.390	0.188
S3	0.875	0.437

Data are the average of 2 determinations.

Ultracentrifugation at $100,000 \times g$ eliminates all membranous material to leave only cytosolic components in supernatant sample, S3. The cytosolic fraction (S3) had the highest overall specific enzyme activity (table 2). The enzyme activity in the pellets was minimal. These data indicate that the peptidase activity is not membrane-bound, but is present in the cytosol. The most probable mechanism of dipeptide utilization involves the extracellular release of the cytosolic peptidase during culture to allow the gradual hydrolysis of the dipeptide in the medium. The gradual release of glutamine from the dipeptide can be compared to the strategy of a fed-batch culture in which glutamine is continuously fed into the culture but maintained at a low concentration. In both cases, the growth yield from carbon substrates was high, thus maintaining an efficient energy metabolism with concomitant low rate of by-product formation. Lower ammonia concentrations released into dipeptide-based medium can be advantageous in attaining high cell yields, particularly for cells that are sensitive to ammonia toxicity.

ACKNOWLEDGEMENT

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EDITOR'S NOTE

The dipeptides are available from Life Technologies as GLUTAMAX™ I (ala-gly, Cat. No. 35050) and GLUTAMAX II (gly-gln, Cat. No. 25060). We have found that the best antibody production results from growing hybridoma cells in GLUTAMAX I and then switching to GLUTAMAX II when cells reach high density. Since hybridoma cells grow poorly in GLUTAMAX II, they expend their energy producing antibodies.

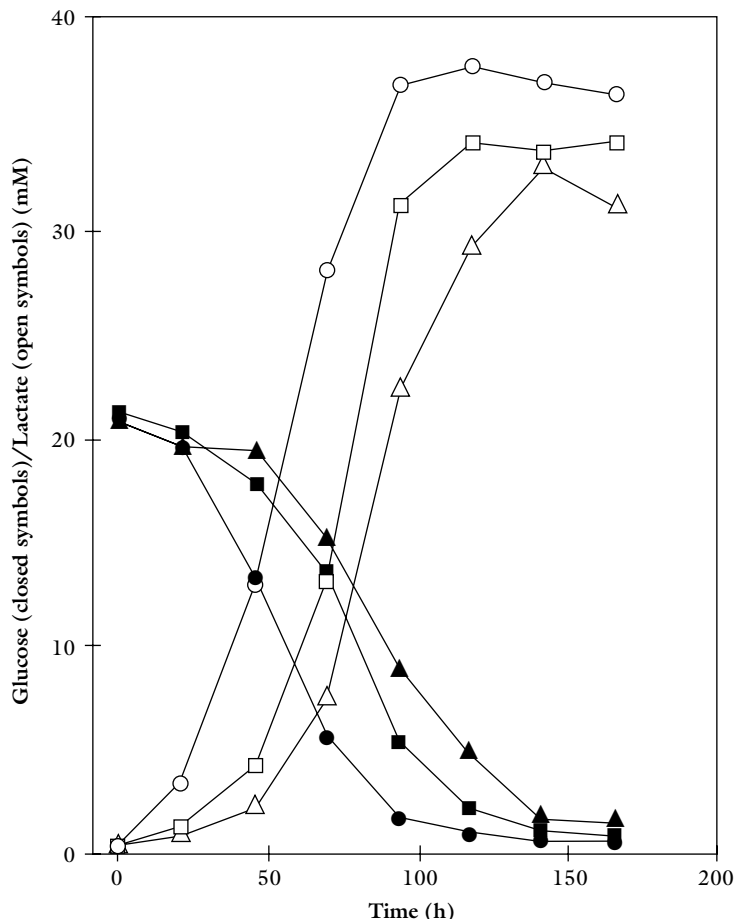
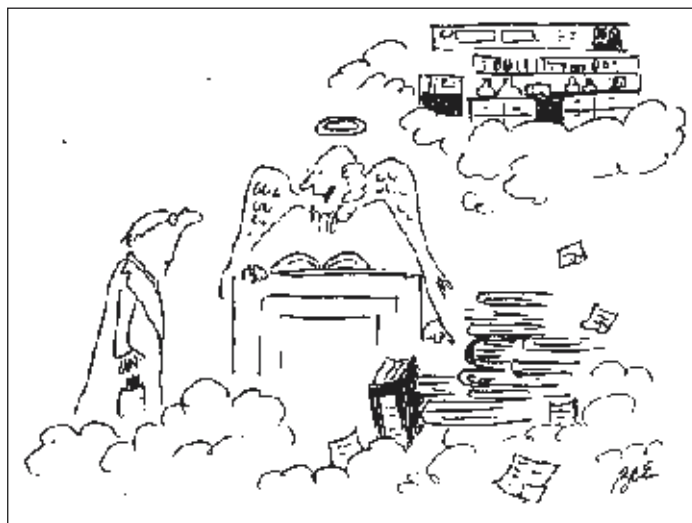


FIGURE 5. Glucose and lactate concentrations in culture. Cells were cultured in D-MEM containing 6 mM gln (●, ○), 6 mM ala-gln (■, □), or 20 mM gly-gln (▲, △).



*"You get a brand new lab with tons of equipment.
But first, deal with the paperwork."*

LOW IgG FBS: APPLICATIONS TO MONOCLONAL ANTIBODY AND VACCINE PRODUCTION

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Fetal bovine serum (FBS) has long been used as a supplement for tissue culture media, supplying growth factors and attachment factors essential for the culturing and proliferation of cells *in vitro*. The production of biological agents [*i.e.*, monoclonal antibodies (MAbs)] from cell culture has highlighted some benefits and drawbacks in using FBS. Hybridomas that cannot be cultured under serum-free conditions have to be cultured in the presence of FBS rather than other sera because of their high concentration of immunoglobulins (1 to 5 mg/ml in newborn calf serum), which would contaminate the secreted MAbs. FBS with its generally low concentration of immunoglobulins (100 to 300 µg/ml) is therefore very important for *in vitro* MAb production. The presence of even low levels of immunoglobulins, in particular IgG, has made the downstream purification of MAbs difficult and is one factor that has driven the need to culture under serum- or protein-free conditions. Protocols for bovine IgG removal and purification of MAbs produced *in vitro* have been developed (1). However, they tend to be tedious and their efficiency depends greatly on the characteristics of the MAb being purified.

The IgG present in the FBS has been shown to interfere with certain animal vaccines produced *in vitro* [*i.e.*, bovine viral diarrhoea (BVD) virus vaccine]. The antibodies present in the FBS neutralize the BVD virus by causing the viral particles to fall apart when the antibody binds to the coat protein of the virus (2,3).

Low IgG FBS (<20 µg/ml) is collected commercially; however, its collection has several problems. For example, it must be collected before or shortly after IgG production has commenced at ~120 to 130 days after gestation (4,5), and the collected blood/serum requires strict segregation to avoid possible contamination with serum that has high IgG levels. The same is true to a certain extent for BVD

antibody "free" FBS, which requires the prescreening of FBS batches in order to find a batch with a suitable low antibody titer. The results reported here describe GIBCO BRL Low IgG FBS (<5 µg/ml, Cat. No. 16250) that also has a very low BVD antibody titer, which eliminates the need for any prescreening of the FBS for IgG or BVD antibody levels. The level of other viral antibodies present in the low IgG FBS is also expected to be very low, although further investigation is necessary to confirm this expectation.

METHODS

Cell culture. All cell culture experiments used GIBCO BRL D-MEM/F12 supplemented with fresh 1% (v/v) GIBCO BRL L-glutamine at the range of FBS concentrations indicated. All the cell culture plasticware was supplied by NUNC. The cell lines were maintained as recommended by the ATCC. Cells were either in log phase or subconfluent prior to use. In the cloning experiments, clones were scored positive using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). If the duplicate cell counts from any flask varied >10%, fresh samples were taken for counting. The cells were cultured as follows.

Mouse myeloma cell line, Sp2, was cultured in medium containing 5% (v/v) FBS. The cells were seeded at 5×10^4 cells/ml in 25-cm² flasks, total volume 10 ml, and incubated at 37°C in a 5% CO₂ atmosphere. Cells were counted using a haemocytometer after 4 days, then subcultured into fresh growth medium at a split ratio of 1:10 and counted 3 days later.

Mouse x mouse hybridoma cells, MH1, were in log phase immediately prior to use in the cloning assay. The cells were washed once in D-MEM/F-12 without serum and serially diluted to a final cell density of 10 cells/ml in medium plus 10% (v/v) test FBS. 0.2 ml of cells were inoculated into 96-well plates using a multichannel pipette, *i.e.*, 2 cells/well. Wells

TABLE 1. Removal of IgG from FBS.

Parameter	Initial Level	Post IgG Removal
IgG	227 µg/ml	1.6 µg/ml
Endotoxin	2 Eu/ml	3 Eu/ml
Total protein	3.78 g/dl	3.58 g/dl

containing clones were scored positive using MTT. All dilution steps were made in D-MEM/F-12 without serum. The relative cloning efficiency (RCE) was determined by comparing the test serum to a control serum, which was an approved lot of FBS.

Human lymphoblast cell line, IM9, was cultured in medium supplemented with 5% (v/v) FBS at 1.2×10^5 cells/ml in 25-cm² flasks. The cells were cultured, counted, and subcultured under identical conditions to the Sp2 cells.

African green monkey kidney fibroblasts, Vero, were cultured in medium supplemented with 5% (v/v) test FBS at 2.5×10^5 cells in 25-cm² flasks. The total volume of the growth medium per flask was 10 ml. After a 5-day growth period, cells were trypsinized and counted using a haemocytometer. The cells were inoculated into 10 ml of fresh growth medium at 2.5×10^5 cells/flask and cultured as described previously.

BVD virus antibody assay. This assay was carried out by the Ministry of Agriculture, Fisheries and Food (MAFF), at its Central Veterinary Laboratory, Weybridge, England. A known "BVD antibody negative" FBS (lowest BVD Ab obtainable) was used as a control. Each sample was tested in quadruplicate. Briefly, the FBS samples were tested by adding 15 µl of FBS to four complete rows of a 96-well plate. BVD virus was titrated into wells in 10-fold dilutions. Samples were incubated for 60 min, the indicator cells (bovine turbinate) were added, and the incubation continued. After 4 days, the plates were observed and evaluated for cytopathic effect.

TABLE 2. Cloning efficiencies in low IgG FBS.

Serum	RCE
Low IgG FBS, lot 1	1.04
Low IgG FBS, lot 2	1.05

MH1 cells were cultured and assayed as described in *Methods*.

TABLE 3. Cell growth assays.

	Passage 1	Passage 2	Passage 3
Sp2 cells			
Initial FBS	13.6	13.8	13.2
GIBCO BRL Low IgG FBS	13.5	13.7	12.6
Supplier A Low IgG FBS	13.5	14.0	13.7
IM9 cells			
Initial FBS	9.6	9.9	10.8
GIBCO BRL Low IgG FBS	9.7	9.7	10.5
Supplier A Low IgG FBS	9.8	8.6	8.4

Results are the mean counts from duplicate flasks (n = 4, SD <9%) expressed as 10⁵ cells/ml.

This assay determines the amount of virus that can be neutralized by the antibody present in the FBS sample. The titer is the last dilution of virus reached that still gave a cytopathic effect expressed as log, *i.e.*, $5 = 10^5$ (a dilution of 1:100,000). A decrease of titer indicates a higher amount of BVD virus antibody present in the serum.

Biochemical analysis. This was carried out at Glasgow University School of Veterinary Department of Clinical Biochemistry on a MIRA biochemical analyzer (Roche Diagnostics).

Protein and endotoxin measurement. Protein was determined using the Biuret method (8). Endotoxin was determined using the limulus amoebocyte lysate gel-clotting procedure (9).

IgG levels in FBS. The level of IgG was determined by ELISA (6,7). The antibody used in the ELISA assay is directed against the heavy chain of the immunoglobulin molecule; thus, low IgG FBS is probably very low in IgM, as well as IgG.

RESULTS

To determine if the IgG could be efficiently removed from FBS, the IgG levels present in the FBS were measured before and after the IgG removal process (table 1). These results indicated that IgG can be removed effectively without endangering the sterility of the FBS.

Biological performance. In order to ensure that factors critical for cell growth and cell attachment were not removed, or levels substantially reduced, a series of biological performance assays including cloning, growth, and cell attachment were carried out on the FBS before and after IgG removal.

TABLE 4. Growth of adherent Vero cells.

Serum	Passage 1	Passage 2
Initial FBS	6.4	8.3
GIBCO BRL Low IgG FBS	6.3	8.2
Supplier A Low IgG FBS	6.5	8.5

Results are the mean counts from duplicate flasks (n = 4, SD <7%) expressed as 10⁶ cells/flasks.

One of the most sensitive of cell growth assays is the cloning assay. The cloning results reported in table 2 were typical of low IgG FBS. The removal of the IgG from FBS had no deleterious effects on the cloning of a mouse hybridoma cell line.

Growth assays were also performed on several cell types over several passages (table 3). No difference in cell number was observed after IgG removal even after several passages.

To determine if the IgG removal process removed any essential cell attachment factors, adherent Vero cells were used (table 4). The results indicated that no critical depletion of any attachment factors occurred in the FBS due to IgG removal. The removal of IgG from the FBS did not adversely affect cell growth (suspension cell lines), cloning (mouse hybridoma), or cell attachment and subsequent cell growth (adherent cell lines).

Biochemical analysis. FBS samples before and after IgG removal were analyzed for a wide variety of components (figure 1). This analysis included enzymes, glucose, protein (albumin), lipids, and metal ions. The major biochemical parameters were not affected by the removal of the IgG.

BVD virus antibody status. FBS samples before and after IgG removal were analyzed to determine the BVD virus antibody titer (table 5). The BVD virus titer increased due to the IgG being removed from the FBS. The low IgG FBS sample produced a higher viral titer than the control FBS used by the MAFF (designated BVD virus antibody “free”) due to the

TABLE 5. BVD virus titer in FBS.

Serum	Viral Titer
MAFF control FBS (BVD virus antibody “free”)	5.75
Initial FBS	4.00
GIBCO BRL Low IgG FBS	6.25
Supplier A Low IgG FBS	4.00

absence of any neutralizing effects caused by the presence of BVD virus antibodies.

DISCUSSION

Low IgG FBS has had its IgG level substantially reduced by a fully validated proprietary removal process. No changes were observed in the biological performance of the FBS in cell culture assays after the removal of the IgG, even after multiple passages. The removal of the IgG from the FBS had no significant effect on the biochemical profile of the serum.

The low IgG levels (<5 µg/ml) will benefit researchers currently growing hybridomas in normal FBS, which has IgG in the range 100 to 300 µg/ml. Culturing hybridoma cells in the low IgG FBS will improve the purity of the secreted MAb. Assuming 10% FBS (v/v) in the medium, the level of contaminating bovine IgG from a hybridoma secreting 50 µg/ml of MAb into the culture medium would be 1% for the Low IgG FBS (worst case) and 20% for normal FBS (best case). The Low IgG FBS will be a useful cell culture supplement in the culturing of serum-dependent hybridomas.

Another benefit of the low IgG FBS is the high BVD viral titer. This will be of great value to those researchers involved in *in vitro* animal vaccine production where contaminating bovine IgG has been a problem.

The alternative to processing the FBS after collection to remove IgG is collecting low IgG FBS before or shortly after IgG production has commenced in the fetus. This event occurs ~120 to 130 days after gestation (4,5)—midway through the second trimester, when the fetus size is relatively small. The volume of serum obtained would be considerably less than that obtained from a fetus that was late third trimester. The volume of serum collected under such a regime is restricted and has to be carefully segregated to avoid possible contamination with BVD virus antibody positive serum. The Low IgG FBS described here does not suffer from such drawbacks.

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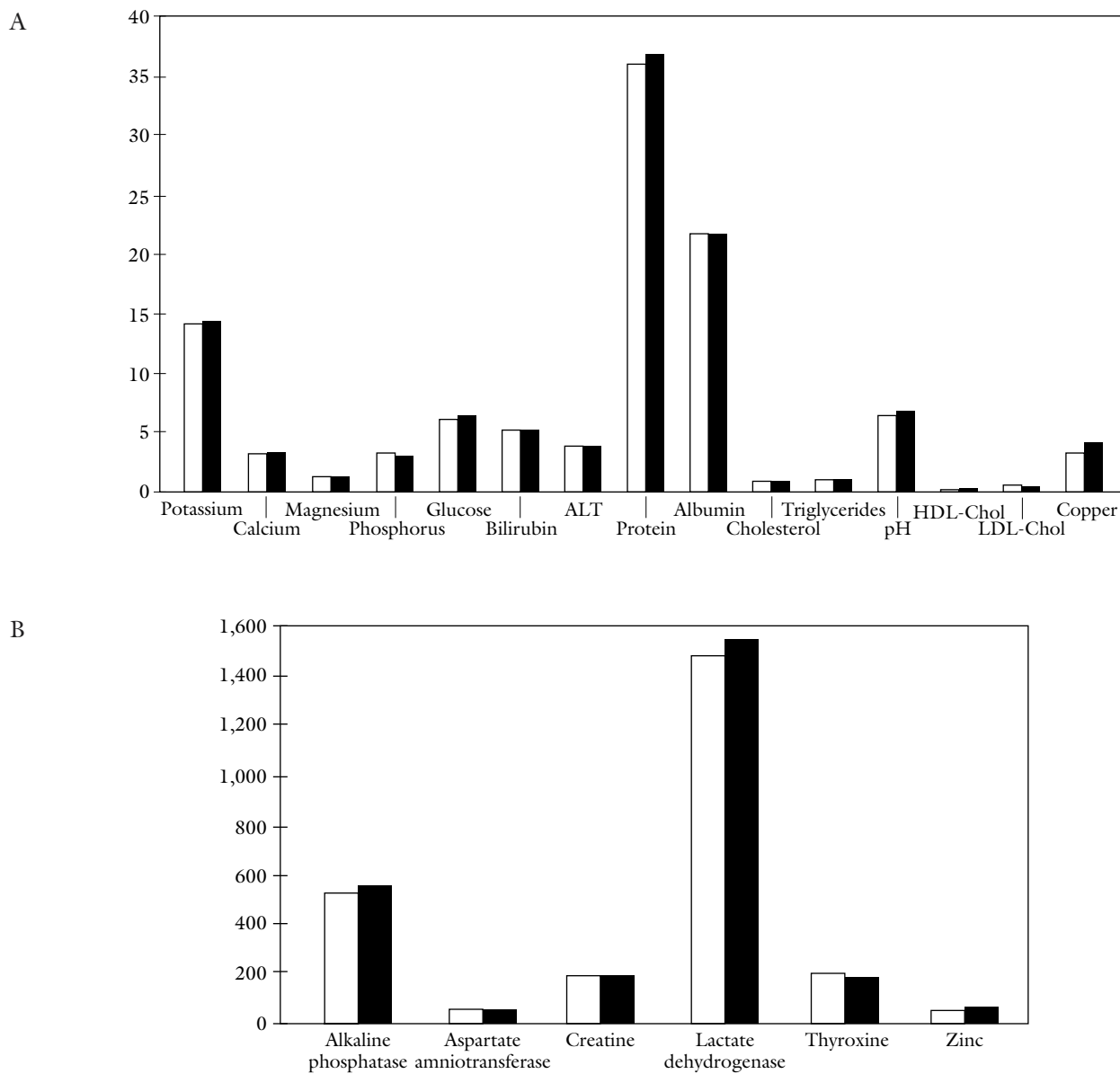


FIGURE 1. Biochemical analysis. Panel A. Sodium, potassium, calcium, magnesium, phosphorus, glucose, bilirubin, cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol concentrations are reported as mM. Protein and albumin are g/l and copper is μM . Panel B. Alkaline phosphatase, aspartate aminotransferase, and lactate dehydrogenase concentrations are reported as IU/l. Thyroxine is nM, and zinc and creatine are μM . Before IgG removal (■), after IgG removal (□).

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A NEW EXPRESSION MEDIUM FOR *AGROBACTERIUM TUMEFACIENS* FOLLOWING ELECTROPORATION

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A *grobacterium*-mediated transformation is one of the most common procedures to establish transgenic plants, especially in dicot plants (1). Introducing DNA into *Agrobacterium* is conventionally achieved by the triparental mating procedure (2). Recently, electroporation was found to be superior to the triparental mating procedure in the time required to introduce foreign DNA into *Agrobacterium* and the elimination of contamination by *E. coli* strains (3).

To achieve a high electrotransformation efficiency, a period of expression is required after transformation. The transformation efficiency of *E. coli* strains has been significantly improved by using the expression medium S.O.C. broth (4). Although many media have been used to grow *Agrobacterium* cells, no defined expression medium has been described for *Agrobacterium* cells that have been transformed using electroporation. In this paper, YM broth is used as an expression medium to increase the transformation efficiency of *Agrobacterium tumefaciens* LBA4404 cells.

Electrocompetent cells of *Agrobacterium tumefaciens* LBA4404 (Cat. No. 18313) were from Life Technologies, Inc. Electroporation was performed at a field strength of 16.7 kV/cm using the GIBCO BRL CELL-PORATOR® Electroporation System and Voltage Booster or

the *E. coli* Pulser. The expression media were LB, S.O.C., M9, EMC, and YM broth (table 1).

Transformants were obtained using all of the expression media tested (table 2). However, the transformation efficiency was significantly affected by the expression medium. The highest transformation efficiency ($>1 \times 10^7$ cfu/ μ g) was obtained using YM broth and YM plates. YM broth consistently showed an increase of 2- to 5-fold in the transformation efficiency of *Agrobacterium tumefaciens* LBA4404 compared to enriched media such as S.O.C. broth and EMC broth. The increase was 10- to 20-fold compared to LB broth. This result indicated that *Agrobacterium tumefaciens* cells were different from *E. coli* cells and required YM broth for the optimized expression using electroporation. Moreover, using YM plates instead of LB plates increased the transformation efficiency about 1.5- to 2-fold, repeatedly. Therefore, an increase in the transformation efficiency of 40-fold was achieved by using both YM broth and YM plates compared to the efficiency using LB broth and LB plates. In addition, the transformed colonies were larger when YM medium rather than LB medium was used.

TABLE 1. Composition of expression medium.

Medium	Components
LB broth	1% bactotryptone, 0.5% yeast extract, 10 mM NaCl (pH 7.0)
S.O.C. broth	2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM (MgCl ₂ ·6H ₂ O+MgSO ₄ ·7H ₂ O), 20 mM glucose (pH 7.0)
EMC broth	2% bactotryptone, 1% yeast extract, 10 mM NaCl, 2.5 mM KCl, 1.5% succinic acid (pH 7.0)
M9 broth	1 mM MgSO ₄ ·7H ₂ O, 1.9 mM NH ₄ Cl, 4.2 mM NaH ₂ PO ₄ , 2.5 mM K ₂ HPO ₄ , 0.4% glucose (pH 7.0)
YM broth	0.04% yeast extract, 1% mannitol, 1.7 mM NaCl, 0.8 mM MgSO ₄ ·7H ₂ O, 2.2 mM K ₂ HPO ₄ ·3H ₂ O (pH 7.0)

TABLE 2. Effect of expression medium on the transformation efficiency of *Agrobacterium tumefaciens* LBA4404.

Expression Medium	Transformation Efficiency (cfu/ μ g $\times 10^6$)	
	YM Plate	LB Plate
YM broth	10	6.3
S.O.C. broth	7.1	3.9
LB broth	0.96	0.25
EMC broth	5.3	3.2
M9 broth	0.36	0.38

Transformation was performed by electroporating 1 ng of pBI121 plasmid DNA with 20 μ l of electrocompetent cells in a microelectroporation chamber at a field strength of 16.7 kV/cm. 10 μ l of the electroporated mixture was diluted to 1.0 ml with different expression media, cultured at 30°C for 3 h, and plated on either a YM or LB plate containing 1.5% agar with 50 μ g/ml kanamycin and 100 μ g/ml streptomycin. The plates were incubated at 30°C for 2 days.

YM medium is one medium used for soil-borne microorganisms such as *Rhizobium* spp. *Rhizobium* cells do not grow well in a rich medium or at high temperatures (5). *Agrobacterium tumefaciens* also is a soil-borne microorganism. Therefore, it is not surprising that YM broth is a better expression medium than any of the other media examined.

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EDITOR'S NOTE

YM broth is now available from Life Technologies (Cat. No. 10090)



How does electroporation compare to triparental mating for introducing recombinant plasmids into *A. tumefaciens*?

For triparental mating, three bacteria are cultured together: an *E. coli* strain containing a mobilization plasmid bearing the transfer genes, an *E. coli* strain containing the recombinant Ti plasmid, and the recipient *A. tumefaciens*. Transformed *Agrobacterium* cells are selected by several rounds of plating on medium containing antibiotics to decrease the risk of *E. coli* contamination. For electroporation, the recombinant plasmid is transformed directly into the *A. tumefaciens* cells. Electroporation has fewer steps, is less time consuming, and eliminates the risk of *E. coli* contamination.

FASTCHECK™ NUCLEIC ACID QUANTIFICATION SYSTEM

Methods to quantitate small amounts or low concentrations of DNA and RNA in solution either are time consuming, involve instrumentation, or are subject to interference by contaminants commonly found in nucleic acid samples. To overcome these problems, the FASTCHECK™ Nucleic Acid Quantification System has been designed for rapid detection and quantification of nanogram to picogram amounts of double-stranded (ds) and single-stranded (ss) nucleic acids. It is suited to quantify residual nucleic acids in materials such as proteinaceous samples derived from recombinant DNA techniques. For detection, the sample is immobilized to a membrane on a dipstick. The FASTCHECK Reagent is a modified alkaline phosphatase that binds specifically to nucleic acids (1). The signal is

detected using BCIP/NBT and quantitated by comparison to a standard. In this paper, the sensitivity and specificity of the FASTCHECK system are examined.

METHODS

Determination of nucleic acid amounts. Nucleic acid samples (1 µl) were spotted on the membrane portion of the dipstick (2.0 × 0.6 cm). After fixing the nucleic acid sample by drying with a heat gun (3-4 inches away) for 30 s and then exposing to UV light from a handheld UV lamp (3-4 inches away) for 30 s (0.01 Joule/cm², or 1 × 10⁴ µW · s/cm²), the sample strip was processed as follows:

1. Blocked for 5 min in FASTCHECK Buffer A;
2. Incubated for 10 min with FASTCHECK reagent (diluted in FASTCHECK Buffer B);
3. Washed for 1 min in FASTCHECK Buffer B;

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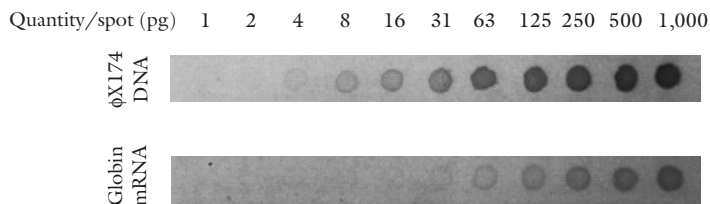


FIGURE 1. FASTCHECK System detection of nucleic acids. 1 μ l of ss ϕ X174 DNA or rabbit globin mRNA was spotted on a membrane and detected with the FASTCHECK System.

4. Washed for 1 min in FASTCHECK Buffer C;
5. Incubated for 4 min with NBT/BCIP solution in FASTCHECK Buffer C.

The signal of the unknown sample was compared to standard (sample of known concentration processed in parallel) or to a standard strip (figure 1) to estimate the amount of unknown nucleic acid. Calf thymus DNA, λ DNA, ϕ X174 DNA, M13mp18(RF) DNA, pUC18 DNA, PCR products, rabbit globin mRNA, and 16S/23S rRNA were analyzed with the FASTCHECK System. Nucleic acid concentrations were determined by A_{260} measurements before they were diluted and assayed with the FASTCHECK System. All FASTCHECK signal intensities were determined by reflective densitometry.

Testing potential interfering agents. M13mp19(+) DNA in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.5)] was compared

with the same DNA mixed with: eluant from agarose and polyacrylamide gels (both were in Tris/borate/EDTA buffer); 0.1, 1, 10, or 100 mg/ml bovine serum albumin (BSA); 0.1% or 1% SDS; 20 μ g/ml glycogen; and 5 or 9 mM dNTP. A serial dilution of DNA samples from 100 to 1 pg/ μ l was compared for impurity interference.

Differentiation of DNA and RNA. To detect and quantify the amount of DNA only or RNA only in a sample containing both DNA and RNA, 3 FASTCHECK dipsticks were spotted with 1 ng of ds M13mp18 DNA and 1 ng of an RNA Ladder. One sample was treated with RNase A, one sample was treated with DNase I, and the control was not treated before FASTCHECK detection. For DNA, the sample was incubated in 5 μ g/ml RNase A (in water) at 37°C for 20 min. For RNA, the sample was incubated in 100 μ g/ml DNase I [in 50 mM Tris-HCl (pH 7), 10 mM $MgCl_2$] at 37°C for 20 min. All 3 dipsticks were rinsed with water and processed in the FASTCHECK solutions.

RESULTS AND DISCUSSION

The FASTCHECK System detected picogram amounts of DNA (1 to 1,000 pg) or RNA (10 to 1,000 pg) (figures 1 and 2). DNA >200 bp bound to the dipstick membrane. Between 1 and 50 kb, the signal intensity did not depend on DNA size (figure 3). Between 200 and 1,000 bp, there was increasing signal intensity with increasing size. Two of three ss DNAs gave less relative intensity compared to equal amounts of their ds DNA forms (table 1). Two RNA species (message and ribosomal) resulted in signal intensities of approximately 60% of the

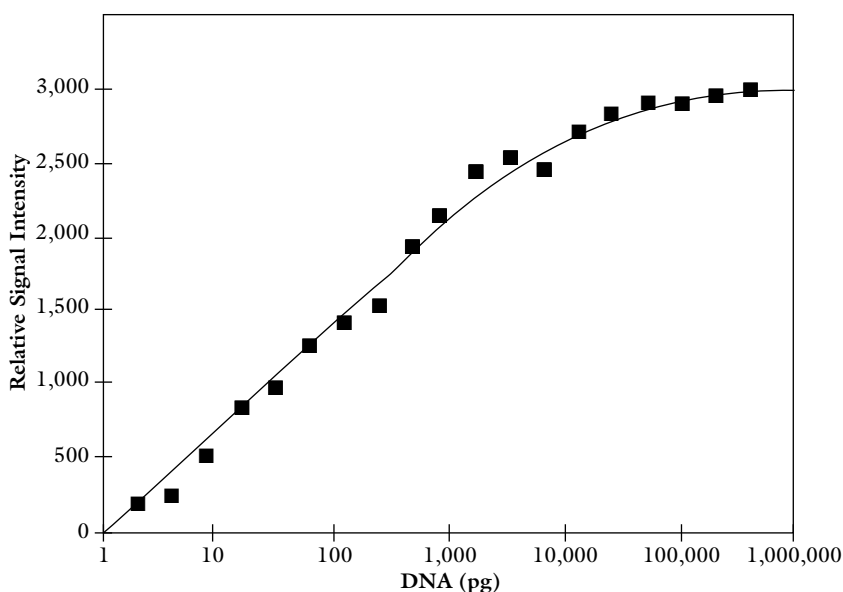


FIGURE 2. The detection range of the FASTCHECK System. ds calf thymus DNA was spotted in 1 μ l and detected by the FASTCHECK System.

TABLE 1. Relative signal intensity of nucleic acids. For each DNA, 250 pg were spotted. The relative signal intensity was determined by reflective densitometry.

Nucleic Acid	Relative Signal Intensity
λ DNA (ds)	107
Calf thymus DNA (ds)	104
ϕ X174 DNA (ds)	92
M13mp18 DNA (ds)	96
pUC18 DNA (ds)	99
λ DNA (ss)	80
Calf thymus DNA (ss)	75
ϕ X174 (ss)	101
16/23S rRNA	69
Rabbit globin mRNA	57

signal of ss ϕ X174 DNA at the same concentration (table 1).

The FASTCHECK System is more sensitive than other methods used to quantitate nucleic acids. The UV absorption method requires several hundred nanograms of nucleic acid in 1 to 2 ml of solution. Fluorescent methods (ethidium bromide or Hoechst stain) need nanograms of DNA in 1 to 2 ml of solution (2,3).

The FASTCHECK System signal was not altered by the most commonly encountered substances that contaminate nucleic acid samples, such as gel matrices, proteins, nucleotides, and detergent (table 2). tRNA had less than 0.5% signal intensity of equivalent amount of DNA (data not shown), potentially allowing for quantitation of DNA containing tRNA from co-precipitation. Proteins and nucleotides do interfere with DNA detection by methods such as UV absorption. Detergents (SDS >0.01%) interfere with fluorescent methods.

Large amounts of protein (>10 mg/ml BSA) did reduce the signal compared to the control. This may be due to the piling up of protein on the nucleic acid sample, decreasing the nucleic acid accessibility to detection. Some complex samples (*i.e.*, whole sera) may have other negative effects at one to several milligrams of protein per milliliter. In this case, sample treatment may be required to reduce the protein present. Sodium iodide extraction was found to be an effective and simple method to remove protein interference (4). DNA samples from 2 to 50 pg in a solution with up to 10 mg/ml (13%) porcine serum were tested with the sodium iodide method. The slightly reduced DNA signal intensity caused by porcine serum was brought back to the control signal

TABLE 2. The effect of common impurities on signal intensity. ss M13mp19 DNA (100 pg) was used. Data are the average of 3 experiments with error ranging from 5% to 10%.

Substance Tested (Level)	Relative Signal Intensity
Control	100%
Agarose	92%
Polyacrylamide	101%
BSA (0.1 to 1 mg/ml)	99%
BSA (10 to 100 mg/ml)	81%
SDS (0.1% to 1%)	112%
Glycogen (20 μ g/ml)	106%
dNTP (5 to 9 mM)	102%

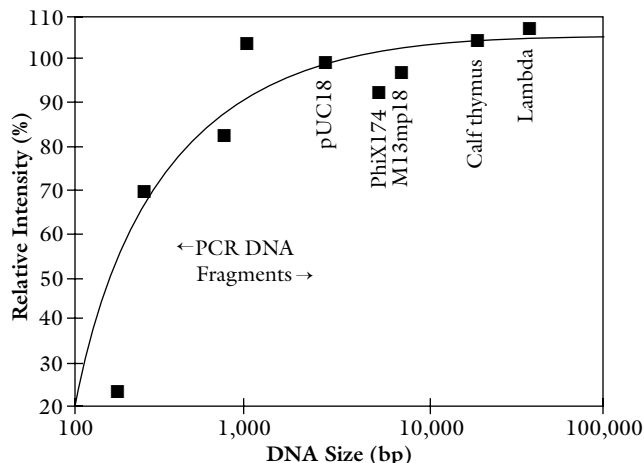


FIGURE 3. The dependence of DNA size and signal intensity. 250 pg of the various ds DNAs were spotted in 1 μ l.

intensity after the sodium iodide treatment (data not shown).

Differentiation of signal due to DNA or RNA can be completed on the dipstick (figure 4). Generally, no interference from RNase A or DNase I was observed on quantitation.

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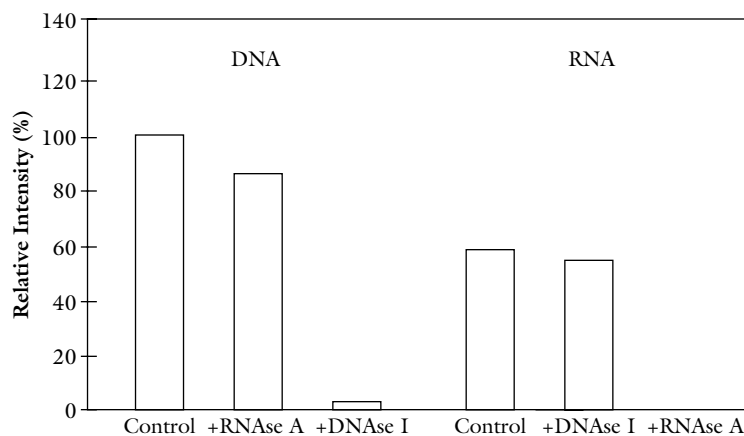


FIGURE 4. Differentiation of DNA and RNA. ds M13mp18 DNA and RNA ladder were spotted, then incubated with DNase I or RNase A and compared to untreated controls.

PROTEINASE K STABILITY

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Proteinase K is a widely used tool for life science investigators. Since proteinase K was first described (1), the conditions used for RNA and DNA preparation have multiplied. Data on the stability of proteinase K in some commonly used buffers have not been investigated. Here we present the results of several stability experiments.

Table 1 reports the half-life ($t_{1/2}$) of proteinase K in a number of commonly used buffers to aid in the design of efficient digestion reactions. The values are for proteinase K without stabilization by substrate and therefore can be considered the minimum stability in each buffer. If the $t_{1/2}$ was >8 h, the percentage of initial activity remaining after an extended incubation was reported. Note that although SDS reduced the half-life of proteinase K, it has

been shown to aid in the digestion of other proteins (2).

There is little data available about the effect of storage conditions on the activity of proteinase K. The effect of repeated freezing and thawing on proteinase K in storage buffer (at 20 mg/ml) was studied. Table 2 lists the percentage of proteinase K activity remaining after several freeze/thaw treatments. The activity was retained even after 12 cycles of freezing/thawing. Although we recommend storage at -20°C , three samples stored at ambient temperature for 16 days retained 98% of the activity as compared to control samples stored at -20°C .

EDITOR'S NOTE

Since proteinase K is stable in solution, it is now available from Life Technologies in the storage buffer tested in this article (Cat. No. 25530). To ensure a high-quality product, all GIBCO BRL Proteinase K is now tested for RNAses. 500 μg of proteinase K is incubated with 1 μg of rabbit globin mRNA at 37°C for 1 and 3 h in REACT[®] 2 (contains 10 mM MgCl_2). At appropriate times, the samples are precipitated and then electrophoresed on a urea-polyacrylamide gel. After staining with methylene blue, the sample lanes are compared to

TABLE 1. Comparison of proteinase K $t_{1/2}$ in commonly used buffers at 50°C and 60°C .

Buffers	Proteinase K (mg/ml)	$t_{1/2}$ at 50°C	$t_{1/2}$ at 60°C
10 mM Tris-HCl (pH 8)	0.1	60 min	5.2 min
100 mM EDTA (pH 8)			
0.5% SDS			
10 mM Tris-HCl (pH 8)	0.1	31 min	4.2 min
25 mM EDTA (pH 8)			
100 mM NaCl			
0.5% SDS			
10 mM Tris-HCl (pH 8)	1.0	3.1 h	24 min
100 mM EDTA (pH 8)			
20 mM NaCl			
1% N-Lauroylsarcosine			
10 mM Tris-HCl (pH 8)	1.0	76% at 24 h	4.1 h
50 mM KCl			
1.5 mM MgCl_2			
0.45% NONIDET [®] P-40			
0.45% TWEEN 20 [®]			

Proteinase K (at 20 mg/ml) was diluted into the chosen buffer that had been pre-equilibrated to 50°C or 60°C and mixed. Immediately a zero time sample was removed to a vial in dry ice. Other time points were taken and the samples immediately frozen on dry ice. All samples were stored at -20°C until assayed. Activity was determined using the azocasein colorimetric protease assay at 37°C (3). A minimum of 2 separate time courses were performed and the average reported.

TABLE 2. Stability of proteinase K under various storage conditions.

Treatment	Number Of Cycles (percent of control)	Activity Remaining
Dry Ice/ 37°C	12	99
$-70^{\circ}\text{C}/4^{\circ}\text{C}$	4	99
Ambient Temp. 16 Days	N/A	98

Proteinase K [20 mg/ml in 10 mM Tris-HCl (pH 7.5), with calcium ion and glycerol as stabilizers] was repeatedly frozen and thawed as follows: Samples were frozen using dry ice for 5 min, and thawed in a 37°C water bath for 2 min. After 12 cycles, the treated samples were assayed for activity along with samples removed just prior to the cold treatment and stored at -20°C . Three samples were placed at -70°C overnight and then 4°C for 8 h. The cycle was then repeated 3 more times. Activity was determined using the azocasein colorimetric protease assay. Values are the average of three separate samples (SD \pm 6%).

control lanes of rabbit globin mRNA. No detectable degradation is seen.

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What are the recommended conditions for proteinase K treatment?

Proteinase K is active over a wide pH range (7.5-12.0) and in the presence of SDS, EDTA, or urea. The usual concentration range for isolating RNA or DNA is 50 to 500 µg/ml in the presence of 1% SDS at 65°C. Since the enzyme is stable at high temperatures, inactivate it by phenol extraction.

ABERRANT MIGRATION PATTERNS OF DNA MOLECULAR SIZE STANDARDS ON AGAROSE GELS

A number of researchers have observed variations in the migration patterns of DNA molecular size standards on agarose gels, including the smearing of bands, the disappearance of certain molecular weight fragments (*i.e.*, 4.3-kb fragment of λ DNA/*Hind* III fragments), and changes in the relative positions of bands (*i.e.*, the ~2-kb fragments of λ DNA/*Hind* III fragments). Because of the intermittent nature of these artifactual banding patterns, there is confusion regarding the correct preparation of DNA standards for electrophoresis. To determine the causes of this aberrant migration, DNA molecular size standards were heated prior to electrophoresis, varying the temperature, incubation time, and ionic strength.

METHODS

All DNA molecular size standards were from Life Technologies, Inc. Electrophoresis buffer was prepared from GIBCO BRL 10X TAE Buffer. 1X TAE buffer contains 40 mM Tris-acetate (pH 8.3) and 1 mM EDTA.

After treatment, DNA standards were made 10 mM EDTA (pH 8), 0.01% bromophenol blue, 0.1% SDS, and 5% glycerol and electrophoresed on 1.0% agarose gels in 1X TAE with a GIBCO BRL HORIZON® 11•14 Horizontal Gel Electrophoresis Apparatus using a GIBCO BRL Model 500 High Current Power Supply. Electrophoresis was performed at ambient temperature (23°C) and constant voltage. Gels were stained by immersion in an ethidium bromide solution (5 µg/ml in 1X TAE) for 15 min and visualized by UV illumination.

RESULTS AND DISCUSSION

Heating DNA ladders or ϕ X174RF DNA/*Hae* III fragments at high temperatures, for extended time, or in low ionic strength can cause denaturation of the double-stranded DNA fragments as evidenced by extra bands or an irregular banding pattern (figure 1). There was a dramatic alteration in the appearance of the bands in the heated versus the same standard without heat treatment. The same effect can be induced even in the presence of

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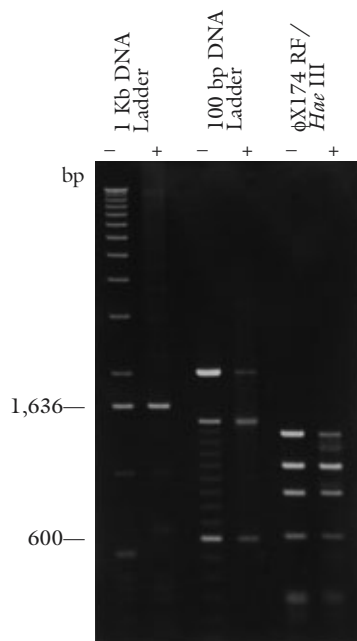


FIGURE 1. The effect of heat treatment on the migration pattern of DNA standards. The DNA was diluted in water, and 100- μ l aliquots were incubated at 65°C for 10 min. 750 ng of heat treated and untreated 1 Kb DNA Ladder and ϕ X174RF/*Hae* III fragments and 200 ng of the 100 bp DNA Ladder were electrophoresed at 85 V until the bromphenol blue migrated 10 cm. Heated standards (+); unheated standards (-).

salt (10 mM Tris-HCl, 0.1 mM EDTA, 5 mM NaCl) with temperatures higher than 65°C or extended incubation at 65°C (data not shown). Alternatively, aberrant migration can be induced during electrophoresis by use of too

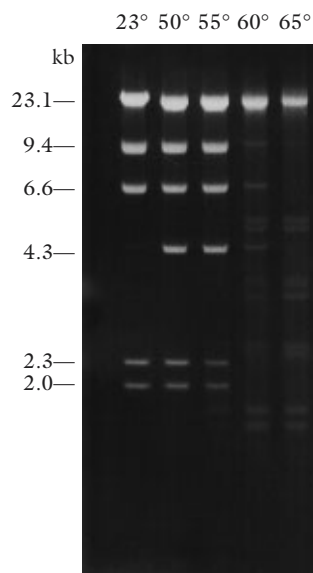


FIGURE 2. The effect of temperature and low ionic strength on λ DNA/*Hind* III fragments. DNA was diluted to 50 μ g/ml with water, and 100- μ l aliquots were incubated 5 min at 23, 50, 55, 60, or 65°C. 500 ng of each sample was electrophoresed at 25 V until the bromphenol blue migrated 12 cm.

high current or inadequate buffering conditions (data not shown).

λ DNA markers do require heating before electrophoresis to dissociate the 12-bp overhangs of the λ cohesive termini (*cos* ends). In a *Hind* III digest of λ , the 23,130-bp and the 4,361-bp fragments contain the left and right termini, respectively. They can anneal to form a larger fragment of 27,491 bp. In unheated λ DNA/*Hind* III fragments, this was reflected by a decrease in the intensity of the ethidium bromide stained band at 4,361 bp and the slower migration of the largest fragment due to an increase in size from 23,130 bp to 27,491 bp (figure 2). However, heating in too low an ionic strength caused strand separation. As shown in figure 2, the 2,322- and 2,027-bp bands denatured at 55°C and a portion of all the fragments denatured at 60°C. At least 20 mM NaCl was required to maintain the native configuration of all the DNA fragments (figure 3). This is consistent with the T_m s of the λ /*Hind* III fragments calculated at 10 mM cation concentration, —63.2°, 62.7°, and 64.3°C for the 2,322-, 2,027- and 590-bp fragments, respectively, and a range of 66.7° to 71.2°C for the larger fragments [$T_m = 81.5^\circ + 16.6 * \log_{10}(\text{cation concentration}) + 0.41 * \%(G+C) - (675/\text{length in bp})$].

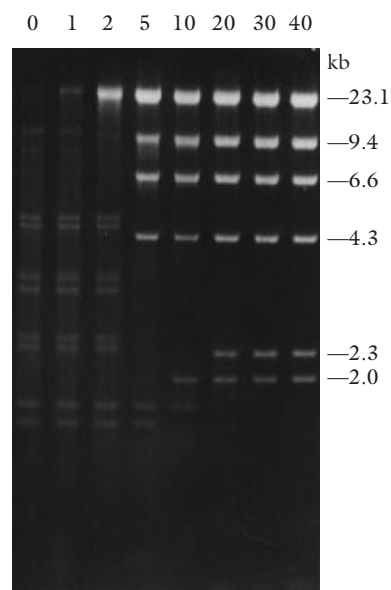


FIGURE 3. The effect of ionic strength on the heat denaturation of λ DNA/*Hind* III fragments. 500 ng λ DNA/*Hind* III fragments in NaCl concentrations of 0, 1, 2, 5, 10, 20, 30, and 40 mM were electrophoresed after heating at 65°C for 10 min.

Determination of size is most accurate when the standards are electrophoresed under the same salt conditions as the samples to which they are compared. Therefore, λ DNA/*Hind* III fragments were heated in REACT[®] buffers to determine if λ *cos* ends could be dissociated at salt concentrations commonly used in restriction digests. A small portion of the *cos* ends of λ DNA/*Hind* III fragments diluted in 1X REACT 2 [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl₂] were separated, while heating the standard in storage buffer [10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5 mM NaCl] at 60-65°C for a maximum of 10 min allowed full *cos* end dissociation and maintained the native double-stranded configuration of the bands (figure 4). Less NaCl was needed to prevent fragment denaturation with 10 mM Tris-HCl than with REACT 2. Alternatively, λ DNA/*Hind* III fragments can be heated in 1X TAE or 1X TBE for use on gels in the respective buffer (data not shown). In addition, DNA standards do not need to be heated immediately before electrophoresis. The DNA can be heated in the chosen buffer, cooled quickly, and stored in final loading buffer at 4°C for more than two months without significant reassociation of the *cos* termini.

In conclusion, λ DNA markers must be heated in at least 20 mM monovalent cation concentration to prevent fragment denaturation, but too high salt concentrations will prevent full dissociation of the λ *cos* ends. DNA ladders and ϕ X174/*Hae* III fragments should not be heated.

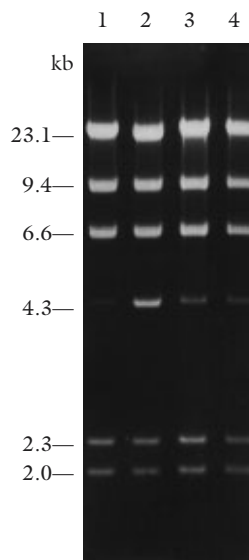


FIGURE 4. The effect of high ionic strength on the dissociation of λ DNA/*Hind* III fragment *cos* ends. DNA was incubated for 10 min at 65°C in varying ionic strengths in a total volume of 100 μ l, and 500 ng of DNA was electrophoresed. Lane 1: 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5 mM NaCl, unheated control; lane 2: 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5 mM NaCl, 65°C; lane 3: 1X REACT 2 [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl₂], 65°C; lane 4: 1X REACT 3 [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂], 65°C.

ACKNOWLEDGEMENTS

We thank Dr. Osama El Badry (Life Technologies, Inc., Training Center) and Dr. Jim Hartley (Life Technologies, Inc., Research and Development) for personal communication of data and helpful discussion.



What can I do to prevent my DNA standards from smearing?

If the ionic strength of the diluted DNA standards is too low, the DNA fragments may denature and electrophorese as a smear. The addition of 10 to 20 mM NaCl will minimize smearing.

COSMID FINGERPRINTING AND SOMATIC CELL HYBRID CHARACTERIZATION USING BIOTINYLATED HUMAN COT-1™ DNA

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Human COT-1 DNA, human placental DNA enriched for the major classes of repetitive elements—*Alu* and *Kpn*—is used to block repetitive sequences in hybridizations where a genomic probe is used, thus alleviating the need to subclone unique sequence away from repetitive elements (1-5). Human COT-1 DNA has been shown to be more effective than total human DNA in Southern blotting and FISH (fluorescence *in situ* hybridization) procedures (1,3,6-11). A notable example of the effectiveness of COT-1 as a suppressor of repetitive elements is its use in combination with *Alu*-PCR probes (6-8), which contain *Alu* termini that can be difficult to suppress. A second use of human COT-1 DNA has been as a ³²P-labeled probe to identify human DNA sequences in various membrane hybridization protocols. These include: more effective identification of human clones in gene libraries derived from human x rodent somatic cell hybrids (11), and identification of DNA sequence overlaps from cosmids or yeast artificial chromosomes (YACs) (12).

In this report, biotinylated human COT-1 DNA (B-COT-1) is used as a hybridization probe in two gene mapping applications. In the first, B-COT-1 is used to identify restriction fragments from a human cosmid clone that contains repetitive elements. In the second application, B-COT-1 DNA is shown to specifically hybridize to the human chromosome complement of a hybrid cell line using FISH.

MATERIALS AND METHODS

Probe. GIBCO BRL Biotinylated Human COT-1 DNA (Cat. No. 25280) or Biotinylated Mouse COT-1 DNA (Cat. No. 18380) was used. The probe was quantitated by Hoechst 33258 fluorometry (13).

Blotting and detection. Cosmid 611, which contains 35 kb of human chromosome 21 cloned into the *Bam*H I site of pWE15, was digested with *Eco*R I, *Bam*H I, and *Pst* I.

0.5 µg of each DNA was electrophoresed on a 0.8% agarose/ TBE gel, denatured, neutralized, transferred in 10X SSC to a nylon membrane, and baked at 80°C for 2 h *in vacuo*. After a 2 h, 65°C prehybridization of membranes in 7% SDS, 0.25 M sodium phosphate (pH 7.2), and 1 mM EDTA, the denatured probe was added in fresh hybridization buffer to a final concentration of 100 ng/ml and hybridized at 65°C for 16 h. Probe was visualized using the PHOTOGENE™ Nucleic Acid Detection System. The membranes were washed to 0.3X SSC at 65°C; blocked with 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% TWEEN 20® (TBS-TWEEN 20), 3% BSA for 1 h at 65°C; incubated with streptavidin-alkaline phosphatase conjugate in TBS-TWEEN 20 (1:1000) for 15 min; washed twice for 15 min each in TBS-TWEEN 20; and washed for 1 h in final wash buffer. One hour after PHOTOGENE Detection Reagent was applied to the membranes, a 2-min exposure on X-ray film was obtained.

FISH. Metaphase spreads of WAV-17 (human chromosome 21 x mouse) hybrid cells were prepared by standard methods (14). Before addition of the B-COT-1 DNA probe, the slides were denatured for 2 min in 70% formamide, 2X SSC at 70°C; dehydrated in 70%, 85%, and 100% ethanol for 1 min each; and air dried. The probe (5 ng/µl, final concentration) was added to the hybridization solution (50% formamide, 2X SSC, 10% dextran sulfate, final concentration), denatured for 5 min at 70°C, placed on ice for 5 min, and applied to the denatured slides that had been prewarmed at 42°C for no more than 2 min. The probe was covered with a glass coverslip and sealed with rubber cement. The slides were incubated for 16 h at 37°C. The coverslips were removed, and the slides were rinsed 3 times for 5 min in 50% formamide, 2X SSC at 45°C; 5 min in 2X SSC at 45°C; 5 min in PN buffer [100 mM sodium phosphate (pH 8), 0.1% NONIDET P-40] at 45°C; and twice for 5 min in room temperature PN buffer. The

probe was detected with 5 µg/ml streptavidin-FITC (Vector) in PN buffer supplemented with 5% nonfat dried milk and 0.02% sodium azide (PNM buffer) for a 20-min incubation, followed by two 2-min washes in PN buffer. The signal was amplified by a 20-min incubation in 5 µg/ml biotinylated anti-streptavidin antibody (Vector) in PNM buffer, rinsed twice for 2 min in PN buffer, incubated in streptavidin-FITC for 20 min, rinsed twice for 2 min in PN buffer, mounted with propidium iodide in antifade solution, and viewed with a Zeiss fluorescence microscope containing an FITC filter set. The slide was photographed using Kodak Ektachrome 400 film without image enhancement.

RESULTS AND DISCUSSION

Detection of restriction fragments containing repetitive elements. In positional cloning strategies, one way to find overlaps between clones in the construction of contigs (contiguous regions of chromosomal DNA that are reconstructed from clone overlaps) is to digest clones with different restriction endonucleases, immobilize the resulting fragments on membranes after electrophoresis, and hybridize them with cognate labeled total, or COT-1 DNA. This procedure, known as fingerprinting, which historically has used a ³²P-labeled probe (12), can be done as effectively with nonradioactive B-COT-1 DNA. A Southern blot with B-COT-1 and chemiluminescent detection was used to identify repetitive element-containing restriction fragments from cosmid clones (figure 1). Independent clones with restriction fragments of the same size that hybridize to the B-COT-1 probe are shared sequences. There are 10 *Eco*R I fragments (including the 8.3-kb vector), and 5 of these hybridized to the B-COT-1 probe (1, 1.7, 3.4, 3.7, and 5.2 kb). Out of the 5 *Bam*H I fragments, three hybridized to the probe. Five out of the eight *Pst* I fragments hybridized to the COT-1 probe.

Identification of human chromosome 21 homologues in a human x mouse somatic cell hybrid cell line. Somatic cell hybrid cell lines are useful in the study of higher genomes, since they allow one to examine chromosomes or parts of chromosomes individually. In the human case, monochromosome hybrids containing each of the 24 chromosomes have been

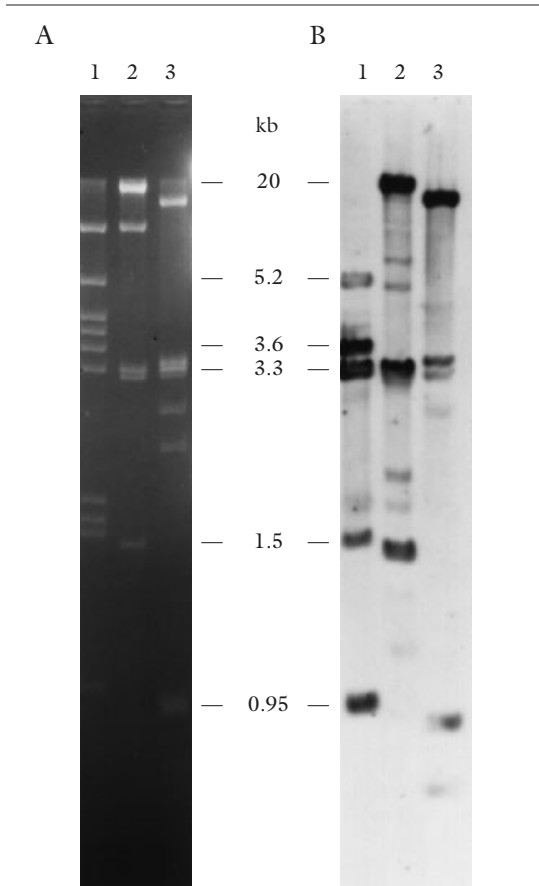


FIGURE 1. Biotinylated human COT-1 detection of repetitive elements on restriction fragments of a human cosmid clone. Panel A. An agarose gel of human chromosome 21 cosmid clone 611 digested with *Eco*R I, *Bam*H I, or *Pst* I (lanes 1-3, respectively). Panel B. A Southern blot on nylon membrane using B-COT-1 DNA, and detected with the PHOTOGENE System.

constructed. The DNA of these chimeric lines can be used to construct gene libraries that contain recombinant molecules from a single human chromosome. Since these cell lines can acquire numerical and structural changes upon repeated passaging in culture, it is important to periodically check a line to be sure it has not changed. There are several alternatives for assessing the quality of a somatic cell hybrid, including Giemsa banding and interspersed repetitive sequence (IRS)-PCR (6,14). Giemsa banding is time consuming and requires extensive cytogenetics experience to be effective (14). Alternatively, IRS-PCR probes (*Alu* or *Kpn*) derived from a cell hybrid DNA and hybridized to a normal human metaphase spread is a less time consuming procedure but technically more challenging (6-8). A third option is using a biotinylated DNA from the species of interest as

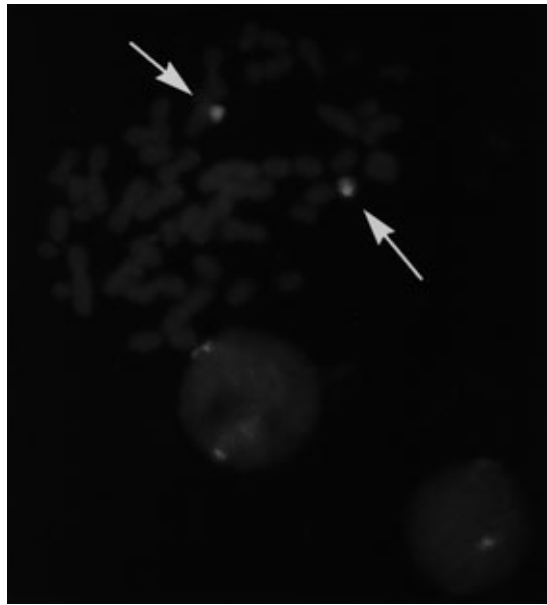


FIGURE 2. Biotinylated human COT-1 detection of 2 human chromosome 21 homologues in a WAV-17 (human x mouse) metaphase spread. Metaphase spreads of WAV-17 cells were prepared and hybridized against a B-COT-1 DNA. The arrows indicate the positions of the 2 human chromosome 21 homologues that were detected.

a FISH probe to assess the content of a cell line. In figure 2, the results of a FISH hybridization of B-COT-1 DNA against the human x mouse hybrid WAV-17 to identify the human component, chromosome 21, provided an unambiguous result. Two small acrocentric chromosomes were decorated by the probe, whereas the mouse chromosomes were unstained. This approach can be used to rapidly check the status of a human x rodent hybrid requiring only a minimal amount of cytogenetics experience.

In conclusion, two applications of the non-radioactive probe, biotinylated Human COT-1 DNA, are described: cosmid fingerprinting, and

somatic cell hybrid analysis. For those laboratories where disposal of radioactive waste is an issue, the use of the PHOTOGENE System in combination with B-COT-1 is an advantage. Also, membranes can be stripped and reprobbed using this method.

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What are the recommended conditions to electrophorese Gibco BRL High Molecular Weight standards?

Electrophorese 200 ng of this standard on a 0.4% agarose gel at 25 V for 12 to 18 h.

RPMI 1640 AND HAM F10 MEDIA MODIFICATIONS FOR CLINICAL CYTOGENETICS TESTS

To obtain a more effective resolution, cytogeneticists use long chromosomes. Longer chromosomes are found in late prophase. Some important problems arise when using long chromosomes. First, all available techniques are more labor intensive than the classical methods. These techniques are only performed when clinical data are well established (for instance, microdeletion syndrome). Apart from this case, when classical methods are applied, there is still some doubt about the result. Second, the higher the resolution, the more difficult the analysis. Also, the mitotic index is sometimes lowered. This paper describes a modification to prepare chromosomes for analysis to minimize some of these problems.

In order to obtain despiralized chromosomes, all known methods use a mitosis blocking agent (1-7). Thymidine can be used for S phase blocking (1) at a concentration of ≥ 0.484 mg/ml (0.2 mM) (5,7). After blocking, the cells are released to synchronize the culture. Late prophasic or prometaphasic chromosomes are harvested at the planned time. We tested a lower concentration of thymidine (0.2 to 0.3 mg/ml) to change the chromosomal structure without blocking. To minimize the number of manipulations, thymidine was added to the medium on the initial culture rather than at the blocking step. This avoids centrifugation, washing, and changing the medium. The altered medium formulation is of interest for clinical cytogenetics tests because better resolution (larger chromosomes) is obtained without any extra effort.

RESULTS

Lymphocyte culture. RPMI 1640 is a classical medium, without thymidine, that is used in cytogenetics laboratories. When RPMI 1640 medium was supplemented with 0.2 to 0.3 mg/ml thymidine, the chromosomes were larger (figure 1). Many prometaphasic chromo-

A

B

C

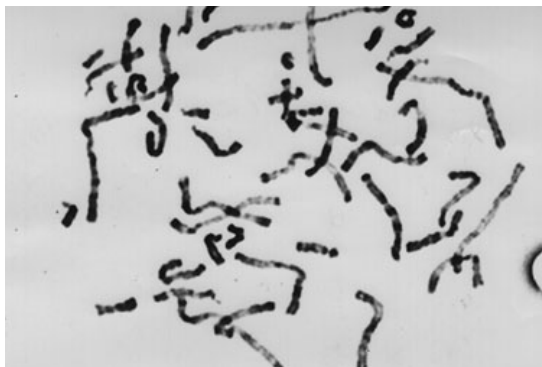
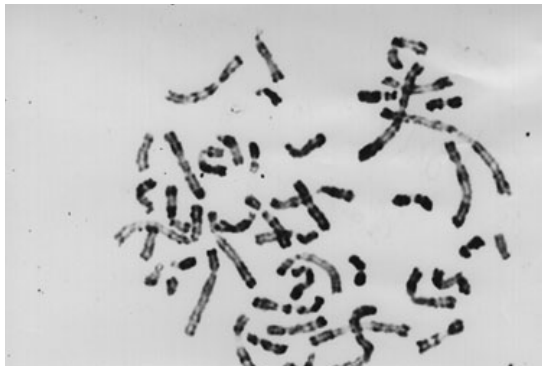


FIGURE 1. Chromosome spreads from lymphocytes treated with thymidine. Panel A. Classical metaphasic chromosomes without thymidine supplementation. Panels B and C. Larger, despiralized chromosomes from cells grown with 0.2 mg/ml thymidine.

somes have been harvested even with the arresting agent (colchicine) used during the last two hours. Below 0.2 mg/ml, no appreciative effect

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TABLE 1. Analysis of RHG chromosome bands.

Chromosome Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Total
	Number of bands counted																								
Medium	17	14	8	11	12	8	10	9	8	12	7	9	9	8	7	7	5	7	5	6	4	5	16	3	207
Medium with thymidine	26	30	18	24	27	21	18	16	16	14	11	17	16	18	15	13	14	10	8	10	8	7	27	5	389

The thymidine concentration was adjusted to 0.2 mg/ml to obtain a karyotype of ~400 bands (RHG banding). A higher resolution can be obtained with the 0.3 mg/ml thymidine.

was observed. Above 0.3 mg/ml, the chromosomes were too despiralized and then the cell cycle was blocked.

Amniocytes culture. Ham F10 medium (containing 0.00073 mg/ml thymidine) was supplemented with thymidine for fibroblasts or amniocyte cultures. With 0.2 to 0.3 mg/ml thymidine, good results were obtained in prenatal diagnosis. The cultures were performed in three different laboratories; two of them did not know the medium modification or the expected results.

In order to obtain prophaseic, prometaphasic, and classical metaphasic chromosomes, two cultures were performed for each patient—one in the classical medium and the other in the thymidine-supplemented medium. The two final pellets were combined and spread on a slide to have all chromosome features on the same slide. The banding patterns were obtained without difficulty on the various mitotic stages (table 1). The longer chromosomes made it possible to count more bands. The well-established variations of banding parameters (as time denaturation for RHG banding) due to different chromatin condensation were not an impediment. However, care should be taken when analyzing breaks and gaps observed on despiralized chromosomes. An image analysis system (Chromoscan) was used to classify the chromosomes.

Over 150 patients have been tested, analyzing 16 or more mitoses per patient (total of 2,400), with similar results. The mitotic index remains the same with the 0.2 to 0.3 mg/ml thymidine supplementation. The number of analyzable cells was increased and the increased

resolution made it easier to observe smaller abnormalities in the chromosomes. Now, all of our standard karyotypes are carried out with the thymidine-supplemented medium.

DISCUSSION

Besides blocking the cell cycle at high concentration, thymidine can be used at a moderate concentration (0.2 to 0.3 mg/ml) to facilitate karyotyping. The moderate excess of thymidine, which does not stop the cell cycle, might increase the duration of the prophaseic and prometaphasic stage leading to chromosome elongation. Although some media are already supplemented by thymidine, the maximal concentration (0.01 mg/ml) is too low to expect the elongation effect. Qu *et al.* (4) also use the moderate thymidine concentration, but their technique is performed as a blocking releasing method, which has more manipulations than our method. It is possible that bromo deoxyuridine, also used to synchronize cells, may have similar results to thymidine at lower concentrations.

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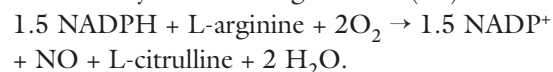
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DIRECT MEASUREMENT OF NITRIC OXIDE FROM OVINE ENDOTHELIUM CULTURED IN ENDOTHELIAL-SFM

Nitric oxide (NO), previously known as endothelium-derived relaxing factor (EDRF) (1), is now recognized to have tremendous significance in numerous regulatory mechanisms (2). NO is a vasodilator, the action of which is mediated via cyclic GMP in target cells (3). It is speculated that gaseous NO diffuses from the site of synthesis to target cells through cell membranes and binds to cytosolic guanylate cyclase to activate the enzyme (4). NO is produced not only by vascular endothelial cells but also by various other cells, including neurons in the central nervous system (5,6), astrocytes (7), macrophages (8), and Kupffer cells (9). Hence, the physiological and pathophysiological role of NO has been extended greatly from a mediator of vascular tone to a mediator of the immune system.

NO is synthesized from L-arginine by nitric oxide synthase (NOS). In endothelial cells, there are two NOS, the constitutive Ca²⁺-dependent NOS and the inducible Ca²⁺-independent NOS (10). It is proposed that NO production by NOS is an oxidation process as indicated by the following reaction (11):



The measurement of NO is important to understand the activity of NOS, as well as the effects of various modulators, on this system. NOS activity is often determined indirectly by incubating radiolabeled L-arginine with the NOS-containing cells and measuring the production rate of L-citrulline (11). However, there is competition between NOS and arginase, which catalyzes the formation of ornithine and urea from arginine (2). Therefore, it is preferable to have evidence for actual NO production. NO determination can be achieved by several methods. For indirect methods, NO is measured in the final metabolites NO²⁻/NO³⁻ or as a spectral change of hemoglobin resulting from a reaction of NO and oxyhemoglobin (8). Unfortunately, these

methods are nonspecific and may render overestimation. By contrast, the direct measurement of gaseous NO by chemiluminescence has been applied by several investigators, demonstrating greater specificity and the ability to quantitate NO as low as 1 pmol/l (12-14).

The production rate of NO depends largely on the availability of substrates, cofactors, and other modulators (11). Hence, it is crucial to control culture conditions to minimize variables that might modulate NOS activity or NO production. Therefore, serum-free medium (SFM) is desirable. Recently, Endothelial-SFM formulated to support the growth of bovine, porcine, ovine, and canine large vessel endothelial cells has been developed (15). This report describes a modified technique to measure head space NO produced by fetal ovine pulmonary endothelial cells cultured in Endothelial-SFM.

Immediately after sacrificing fetal sheep with an excess dose of sodium pentobarbital, the main pulmonary artery was excised. The endothelium was carefully removed by gentle scraping with a scalpel blade, cultured, and characterized as previously described (15). Cultures were expanded for 14 to 16 days, after which time endothelial cells (1×10^6 to 2×10^6) were put in 2 ml of Endothelial-SFM in an 8-ml culture vial and placed in a 37°C water bath. After 30 min, the head space NO gas was measured every 15 min over 3 h, replacing the head space NO gas with an O₂ (95%) + CO₂ (5%) gas mixture. NO in the head space equilibrates with NO in the medium as described by Henry's law. Hence, the partial pressure of NO in the head space and medium is similar. By replacing head space gas with NO-free gas, the medium NO rapidly equilibrates in the entire vial. In our studies using 2 s for withdrawal and replacement, ~90% of the NO in the vial was collected and introduced into the detector. Therefore, the NO measurement includes both the NO in the head space and in the medium. In addition, this intermittent gas collection method allows for both accumulation

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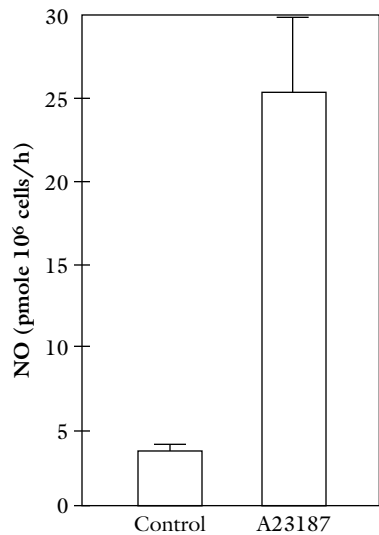


FIGURE 1. NO production by fetal ovine pulmonary endothelium. Cells were cultured in Endothelial-SFM without (control) or with 1 μ M A23187. Values are mean \pm sem (n = 6, p < 0.05).

of measurable NO from a smaller population of cultured endothelium and relatively accurate production rate determination, yet provides good ventilation for the cells and enough time points to observe details during experimental events.

NO and nitrogen oxides were measured using a Model 207B Reduction Chemiluminescence Detector (RCD, Sievers Research Inc.) connected to a vacuum pump (Model E2M-1, Edwards High Vacuum). The sampling port of the RCD was connected to 50 cm of stainless tubing (dead space 0.4 ml), which was surrounded by dry ice as a cool trap. A 25-g needle was fitted to the end of this tubing for sampling from the rubber-capped culture vial. The suction rate of the pump was 15 ml/s and the integration time for the signal was set at 2 s to avoid underestimation. The system was calibrated as follows: 10-, 20-, 50-, and 100- μ l aliquots of the certified calibration NO gas (Matheson, 5 ppm) were obtained from a reservoir using airtight microsyringes and injected into vials with 2 ml of medium. These aliquots were calculated in molar units with correction for ambient temperature and atmospheric pressure. A 10- μ l aliquot of 5 ppm NO gas contains \sim 2 pmol NO at room temperature. The standard curve was linear, with a negligible intercept and a correlation coefficient of 0.99.

Sample gases obtained from the head space of culturing vials were introduced into the RCD in the same way. Calibration of the RCD was repeated following the sample measurements.

Since the RCD signal was integrated via a Hewlett-Packard Integrator (Model 2201), the raw data were total molar amount and then calculated as NO output/million cells/h (pmol/10⁶ cells/h). The measured NO amounts from 2 \times 10⁶ cells over 15 min varied from 1 to 3 pmol in the control period. Over the 3-h incubation, NO output was constant, but there was a tendency for a gradual rise. Endothelial cells in culture produced 3.3 \pm 0.4 pmol/10⁶ cells/h (figure 1). There was a 7-fold increase in NO production upon addition of the calcium ionophore A23187 to the medium. Control experiments demonstrated no detectable NO from culture medium alone (data not shown).

There are a few studies that measured NO from cultured vascular endothelium. Radomski *et al.* reported an NO production rate by porcine aortic endothelial cells of 87 \pm 7 pmol/mg of protein/min (16). Assuming that 10⁸ cells yield 1 mg protein, the NO output was \sim 52 pmol/10⁶ cells/h, which is $>$ 15-fold greater than our control data. However, they used the indirect method based on the turnover ratio of oxyhemoglobin to methemoglobin. NOS activity was measured as 0.37 pmol/min/g tissue for the production rate of NO in the cultured endothelial cells from rat aorta (17). Even assuming 1 g tissue is \sim 10⁹ to 10¹⁰ cells, this value seems much lower than data of the present study and other studies. In cultured macrophages (HD11, and avian MC29-transformed cell line) using a direct chemiluminescence measurement system, the stimulated NO production rate by LPS was 15 to 25 pmol/10⁶ cells/h (12). This is similar to the A23187-simulated NO production reported here.

Because of inconsistent results of NO output among various studies, it is unclear whether the basal NO production determined in the present study is physiologically accurate. The measurement of NO by chemiluminescence yields lower values than do indirect measurements. This could be attributed to either the medium or the measurement system for NO. Since the substrate L-arginine may have a possi-

ble role on the catalytic activity of NOS (18), the level of L-arginine in the medium should be standardized. In this regard, the consistent response to A23187 of sheep endothelial cells in the present study demonstrated the application of SFM in the study of NO.

ACKNOWLEDGEMENTS

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