The University *of* Western Ontario
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: October 14, 2011
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario (UWO) or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biological agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biological agents being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Electronically completed forms are to be submitted to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190 or to jstanle2@uwo.ca) for distribution to the Biohazards Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety.

Please ensure that all questions are fully and clearly answered. Failure to do so will lead to the form being returned, which will cause delays in your approval and frustration for you and your colleagues on the Committee.

If you are re-submitting this form as requested by the Biohazards Subcommittee, please make modifications to the form in bold print, highlighted in yellow. Please re-submit forms electronically.

PRINCIPAL INVESTIGATOR:	**Rebecca Jane Rylett**
DEPARTMENT:	**Molecular Brain Research Group**
ADDRESS:	**Robarts Research Institute**
PHONE NUMBER:	**24078**
EMERGENCY PHONE NUMBER(S):	**519-777-9248**
EMAIL:	**jane.rylett@schulich.uwo.ca**

Location of experimental work to be carried out :

Building :	**Robarts Research Institute**	Room(s):	**3250**
Building :	**Robarts Research Insittute**	Room(s):	**3278**
Building :		Room(s):	

***For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 15.0, Approvals).**

FUNDING AGENCY/AGENCIES: **CIHR**
GRANT TITLE(S): **Effects of Oxidative / Nitrosative Stress and Aging on Cholinergic Neuron Function**
UNDERGRADUATE COURSE NAME(IF APPLICABLE):

List all personnel working under Principal Investigators supervision in this location:

Name	UWO E-mail Address	Date of Biosafety Training
Daisy Wong	**dwong@robarts.ca**	**08-Nov-2010**
Leah Cuddy	**lcuddy@uwo.ca**	**13-Mar-2012**
Shawn Albers	**salbers@uwo.ca**	**21-Sep-2010**
Trevor Morey	**tmorey@uwo.ca**	**06-Feb-2012**
Bettina Kalisch	**bkalisch@uoguelph.ca**	**05-Dec-2011**

Please explain how the biological agents are used in your project and how they are stored and disposed of. The BARF without this description will not be reviewed.

Biological agents used are recombinant DNA plasmids that encode selected genes of interest that are then expressed in cell lines or primary neuron cultures. Plasmid DNA is replicated in E. coli [DH5-alpha] and stored as frozen stocks. Long-term storage is at -80oC. For inactivation, disinfection and disposal, solutions, solid waste such as pipettor tips and surfaces are treated with 1-% bleach for at least one hour. Other solid wastes are autoclaved prior to disposal. These studies are focused on changes in brain chemistry associated with normal aging and degenerative neurological and psychiatric disorders. This provide an assessment of how nerve cells communicate and conditions that promote heathly brain aging, and therapeutic interventions that may be beneficial for treatment of dysfunctions. Experimental models involve cellular and molecular approaches, protein chemistry and function, trafficking of proteins in cells and interactions of cellular constituents and their role in regulation of cell function.

Type-5 Adenovirus encoding choline acetyltransferase are replicated in HEK 293 cells and purified from culture medium, then stored as frozen stocks at -80oC. Culture media from cells transfected with plasmid DNA or transduced with viral stocks are treated with 10% bleach prior to disposal by flushing with a large volume of water. Other contaminated lab wares or surfaces are either treated with bleach or autoclaved prior to disposal. Recombinant adenovirus is used for expression of proteins in primary neuron cultures or neural cell lines.

See Attachment: 1

Please include a ONE page research summary or teaching protocol in lay terms.
Forms with summaries more than one page will not be reviewed.

Age-related neurological and cardiovascular diseases are associated with cholinergic neuron dysfunction and cognitive deficits. Little is known about mechanisms by which age-related changes impact cholinergic function or if this can be prevented or reversed. Some changes are initiated by oxidative and nitrosative stress that increase as pathology progresses, and there is a relationship with altered amyloid precursor protein (APP) metabolism and β-amyloid peptide (Aβ) production. Modified APP/Aβ metabolism is a hallmark of Alzheimer disease, but also a result of cardiovascular disease including ischemia, stroke and hypertension. Age-related increases in Aβ modulate synaptic communication and play a central role in Alzheimer-related synaptic changes. The tissue stress response and generation of reactive oxygen (ROS) and nitrogen species (RNS) in aging brain and early vascular and neuronal disease is attributed partly to increased Aβ. Increased ROS-RNS initiate changes resulting in reversible and irreversible alterations in protein structure and function.

Hypothesis: Oxidative and/or nitrosative stress in brain initiated in part by age and disease-related increases in Aβ or by decreases in antioxidant capacity diminishes ACh synthesis due to post-translational effects on choline acetyltransferase (ChAT) and the choline transporter (CHT).

We will use proteomic, cell biology and in vivo studies to characterize dynamic responses ChAT and CHT to age-related changes. Protein regulation by reactions with ROS and RNS is a critical modulator in physiological and pathological situations. Limited cellular mechanisms are normally in place, such as formation of protein mixed-disulphides with glutathione (GSH), that transiently protect proteins from detrimental effects of ROS-RNS until redox balance is restored. S-Glutathionylation, formed at critical reactive Cys from ROS or RNS, may cause a gain-of-function for some proteins. However, in aging and disease, GSH levels decrease with irreversible oxidation and loss-of-function of vulnerable proteins.

Specific Aim 1: Investigate the potential for ChAT to undergo functional and/or structural changes during aging or when exposed to either oxidative-nitrosative conditions or to soluble Aβ. We will characterize changes to ChAT structure-function in brain of aging mice and APP/presenilin-1 over-expressing Alzheimer mice, and link this to mechanistic studies at cellular and protein levels. We will test oxidative modification of cysteine (Cys) in ChAT in SH-SY5Y cells expressing human ChAT exposed to ROS-RNS or Aβ(1-42), and the effect that this has on ChAT stability and interaction of with other proteins. Purified ChAT will be used to determine accessibility and reactivity of Cys residues, and to assess if Cys residues undergo protective modification with GSH or be irreversibly inactivated.

Specific Aim 2: Determine mechanisms by which ROS-RNS and Aβ alter trafficking and activity of CHT causing decreased choline uptake. We will test if CHT activity and subcellular disposition are altered in cholinergic nerves from young and aging normal and Alzheimer mice. Changes in cell surface CHT density and internalization will be monitored by biotinylation and confocal microscopy in live cells treated with ROS, RNS and Aβ. We will define mechanisms underlying decreased CHT activity in ROS-RNS and Aβ(1-42) treated cells by testing effects on CHT trafficking and subcellular disposition. We will identify Cys modified in CHT by ROS-RNS exposure and functional outcomes in CHT trafficking and dimerization, and choline transport activity and ACh synthesis.

Significance: Our studies will give critical information about the role of age-related or Aβ-induced oxidative stress in cholinergic dysfunction, and the first mechanistic data about how perturbations directly impact key proteins required for ACh synthesis. We define changes consistent with healthy and successful brain aging compared to changes associated with onset of age-related disorders. This will give insight into reversible changes amenable to intervention and identify therapeutic targets for protection of these proteins in aging and disease.

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☒ YES ☐ NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ☐ YES ☒ NO
If YES, please give the name of the species ________
What is the origin of the microorganism(s)? ________

Please describe the risk (if any) of escape and how this will be mitigated:

Please attach the CFIA permit.

Please describe any CFIA permit conditions:

1.2 Please complete the table below:

Full Scientific Name of Biological Agent(s)* (Be specific)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
***E. coli* DH5-alpha**	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**0.25 L**	**Life Technology**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
Type-5 adenovirus	☒ Yes ☐ No	☒ Yes ☐ No	☐ Yes ☒ No	**0.03 L**	**constructs generated in lab of Dr. R. Gros**	☐ 1 ☒ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3

****Please attach a Material Safety Data Sheet or equivalent from the supplier if the bacterium used is not on this link:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

Additional Comments: **See Attachment: 2**

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☒ YES ☐ NO
(If NO, please proceed to Section 3.0)

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☐ Yes ☒ No		Not applicable
Rodent	☒ Yes ☐ No	**brain**	**2010-025**
Non-human primate	☐ Yes ☒ No		
Other (specify)	☐ Yes ☒ No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Containment Level of each cell line	Supplier / Source of cell line(s)
Human	☒ Yes ☐ No	**HEK293** **SH-SY5Y**	**2** **1**	**ATCC** **ATCC**
Rodent	☒ Yes ☐ No	**SN56**	**1**	**Dr S Pasternak**
Non-human primate	☐ Yes ☒ No			
Other (specify)	☐ Yes ☒ No			

Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ☒ 1 ☒ 2 ☐ 2+ ☐ 3

Additional Comments: **See Attachment 3 and 4**

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☐ YES ☒ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/UNKNOWN**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Blood (fraction) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (unpreserved)		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

Additional Comments:

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☒ YES ☐ NO If **NO**, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☒ YES, complete table below ☐ NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transformed or Transfected	Will there be a change due to transformation of the bacteria?	Will there be a change in the pathogenicity of the bacteria after the genetic modification?	What are the consequences due to the transformation of the bacteria?
Ecoli DH5-alpha	**pcDNA3.1 peGFP peYFP peCFP**	**Clontech**	**- choline acetyltransferase - choline transporter - parkin**	**no**	**no**	**replication of plasmid DNA**

**** Please attach a Material Safety Data Sheet or equivalent if available.***
***** Please attach a plasmid map.***
******No Material Safety Data Sheet is required for the following strains of E. coli:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

4.3 Will genetic modification(s) of bacteria and/or cells involving viral vectors be made?
☒ YES, complete table below ☐ NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction
Type 5 adenovirus	**See Attachment: 2**	**See Attachment: 2**	**choline acetyltransferase**	**choline acetyltransferase protein expressed in cells**

**** Please attach a Material Safety Data Sheet or equivalent.***

4.3.1 Will virus be replication defective? ☒ YES ☐ NO

4.3.2 Will virus be infectious to humans or animals? ☐ YES ☒ NO

4.3.3 Will this be expected to increase the containment level required? ☐ YES ☒ NO

5.0 Will genetic sequences from the following be involved?

- HIV ☒ NO ☐ YES, specify
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens ☒ NO ☐ YES, specify
- SV 40 Large T antigen ☒ NO ☐ YES
- E1A oncogene ☐ NO ☒ YES
- Known oncogenes ☒ NO ☐ YES, specify
- Other human or animal pathogen and or their toxins ☒ NO ☐ YES, specify

5.1 Is any work being conducted with prions or prion sequences? ☒ NO ☐ YES

Additional Comments: **See Attachment: 2**

6.0 Human Gene Therapy Trials

6.1 Will human clinical trials be conducted involving a biological agent? ☐ YES ☒ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 7.0

6.2 If YES, please specify which biological agent will be used:
Please attach a full description of the biological agent.

6.3 Will the biological agent be able to replicate in the host? ☐ YES ☐ NO

6.4 How will the biological agent be administered?

6.5 Please give the Health Care Facility where the clinical trial will be conducted:

6.6 Has human ethics approval been obtained? ☐ YES, number: ☐ NO ☐ PENDING

7.0 Animal Experiments

7.1 Will live animals be used? ☒ YES ☐ NO If **NO**, please proceed to section 8.0

7.2 Name of animal species to be used **mouse**

7.3 AUS protocol # **2010-025**

7.4 List the location(s) for the animal experimentation and housing. **housed in MSB, brought to Robarts**

7.5 Will any of the agents listed in section 4.0 be used in live animals
☒ NO ☐ YES, specify:

7.6 Will the agent(s) be shed by the animal:
☐ YES ☒ NO, please justify:

8.0 Use of Animal species with Zoonotic Hazards

8.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? ☐ YES ☒ NO - If **NO**, please proceed to section 9.0

8.2 Will live animals be used? ☐ YES ☐ NO

8.3 If **YES**, please specify the animal(s) used:

♦ Pound source dogs	☐ YES	☐ NO
♦ Pound source cats	☐ YES	☐ NO
♦ Cattle, sheep or goats	☐ YES, species	☐ NO
♦ Non-human primates	☐ YES, species	☐ NO
♦ Wild caught animals	☐ YES, species & colony #	☐ NO
♦ Birds	☐ YES, species	☐ NO
♦ Others (wild or domestic)	☐ YES, specify	☐ NO

8.4 If no live animals are used, please specify the source of the specimens:

9.0 Biological Toxins and Hormones

9.1 Will toxins or hormones of biological origin be used? ☐ YES ☒ NO If **NO**, please proceed to Section 10.0

9.2 If YES, please name the toxin(s) or hormones(s)
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

9.3 What is the LD_{50} (specify species) of the toxin or hormone

9.4 How much of the toxin or hormone is handled at one time*?

9.5 How much of the toxin or hormone is stored*?

9.6 Will any biological toxins or hormones be used in live animals? ☐ YES ☐ NO
If **YES**, Please provide details:

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

Additional Comments: _____

10.0 Insects

10.1 Do you use insects? ☐ YES ☒ NO - If **NO**, please proceed to Section 11.0

10.2 If YES, please give the name of the species.

10.3 What is the origin of the insect?

10.4 What is the life stage of the insect?

10.5 What is your intention? ☐ Initiate and maintain colony, give location:
☐ "One-time" use, give location:

10.6 Please describe the risk (if any) of escape and how this will be mitigated:

10.7 Do you use insects that require a permit from the CFIA permit? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

11.0 Plants

11.1 Do you use plants? ☐ YES ☒ NO - If **NO**, please proceed to Section 12.0

11.2 If YES, please give the name of the species.

11.3 What is the origin of the plant?

11.4 What is the form of the plant (seed, seedling, plant, tree...)?

11.5 What is your intention? ☐ Grow and maintain a crop ☐ "One-time" use

11.6 Do you do any modifications to the plant? ☐ YES ☐ NO
If yes, please describe:

11.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:

11.8 Is the CFIA permit attached? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

12.0 Import Requirements

12.1 Will any of the above agents be imported? ☐ YES, country of origin ☒ NO
If **NO**, please proceed to Section 13.0

12.2 Has an Import Permit been obtained from HC for human pathogens? ☐ YES ☐ NO

12.3 Has an import permit been obtained from CFIA for animal or plant pathogens? ☐ YES ☐ NO

12.4 Has the import permit been sent to OHS? ☐ YES, please provide permit # ☐ NO

13.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biological agents in Sections 1.0 to 9.0 have been trained.

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name Rebecca Jane Rylett ***Date:*** 23 July 2012

14.0 Containment Levels

14.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. ☐ 1 ☒ 2 ☐ 2+ ☐ 3

14.2 Has the facility been certified by OHS for this level of containment?
☒ YES, location and date of most recent biosafety inspection: **24 July 2012**
☐ NO, please certify
☐ NOT REQUIRED for Level 1 containment

14.3 Please indicate permit number (not applicable for first time applicants): **BIO-RRI-0022**

15.0 Procedures to be Followed

15.1 Are additional risk reduction measures necessary beyond containment level 1, 2, 2+ or 3 measures that are unique to these agents? ☐ YES ☒ NO
If **YES** please describe:

15.2 Please outline what will be done if there is an exposure to the biological agents listed such as a needlestick injury or an accidental splash:
Needle stick-express wound to bleed, wash with soap and water for 15 minutes and report to Work Place Health

15.3 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/workplacehealth.html

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name Rebecca Jane Rylett ***Date:*** 23 July 2012

15.4 Additional Comments: _____

16.0 Approvals

1) UWO Biohazards Subcommittee:
SIGNATURE: _____
Date: _____

2) Safety Officer for the University of Western Ontario
SIGNATURE: _____
Date: _____

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: _____
Date: _____

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

Attachment : 1

RYLETT LABORATORY GUIDELINES FOR THE SAFE HANDLING OF ADENOVIRAL VECTORS

ADENOVIRAL EXPERIMENTS --- Standard Operating Procedures:

- Laboratory coats, gloves and safety glasses are worn while handling the adenoviral vectors.

- Adenoviral containing materials are handled inside the biological safety cabinet (BSC) in room 3278.

- All tissue culture work related to adenoviral vectors experiments are conducted inside the BSC.

    - Only materials needed for adenoviral experiments are placed in BSC.

    - All serological pipettes, pipette tips are decontaminated in a virucide (Clidox, Quatricide or freshly prepared 10% household bleach) for 30 minutes prior to discarding into biohazard waste container.

    - Upon completion of work inside the BSC, all work surfaces and equipment used inside the BSC are sprayed with the virucide (Clidox or Quatricide) and then with 70% ethanol and air-dried.

    - All solid waste materials related to the adenoviral experiments are placed in biohazard waste bag and sealed for disposal (i.e. to be autoclaved).

    - Vacuum lines for liquid waste collection are filtered with a HEPA filtered before entering the vacuum system. For aspirated liquid waste, aspirate full-strength bleach through the suction tube into the liquid waste container to the approximate final concentration (1 in 10) and soak for 20-30 minutes and empty entire contents down the drain. Rinse drain and liquid waste flask with 70% ethanol.

- Transportation of adenoviral vector containing materials will be done in plastic containers (50 ml conical tubes) contained inside a leak-proof container.

- All the centrifugation containing adenoviruses is done in swinging bucket rotor which is sealed with aerosol tight screw caps.

In case of adenoviral vector spill outside the BSC, warn everyone in the immediate area contain spill with bleach soaked paper towels and mop spill with paper towels, re-apply bleach and soak for 30 minutes. All waste materials are placed in biohazard bag and area is cleaned again with bleach solution followed by 70% ethanol wash.

Attachment 2: Adenovirus inventory

Virus used for Transduction	Vector	Source of Vector	Gene Transfected	Change that results
Adenovirus	pDC316	Dr. R Gros	GFP	Express GFP
Adenovirus	pDC316	Dr. R Gros	NLS	Express NLS
Adenovirus	pDC316	Dr. R Gros	82kDa ChAT	Express 82kDa ChAT
Adenovirus	pDC316	Dr. R Gros	Mutant R442H	Express Mutant R442H

Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

Office of Biohazard Containment and Safety
Science Branch, CFIA
59 Camelot Drive, Ottawa, Ontario K1A 0Y9
Tel: (613) 221-7068 Fax: (613) 228-6129
Email: ImportZoopath@inspection.gc.ca

Bureau du confinement des biorisques et sécurité
Direction générale des sciences, ACIA
59 promenade Camelot, Ottawa, Ontario K1A 0Y9
Tél: (613) 221-7068 Téléc: (613) 228-6129
Courriel: ImportZoopath@inspection.gc.ca

October 20th, 2009

Ms. Shamila Survery / Mr. Michael Decosimo
Cedarlane Laboratories Ltd
4410 Paletta Court
Burlington, Ontario L7L 5R2

By Facsimile: (289) 288-0020

SUBJECT: Importation of *Escherichia coli* strains

Dear Ms. Survery / Mr. Decosimo:

Our office received your query about the importation of *Escherichia coli* from the American Type Culture Collection (ATCC) located in Manassas, Virginia, United States. The following *Escherichia coli* strains are consider to be level 1 animal pathogens:

- 5K
- 58
- 58-161
- 679
- 1532
- AB284
- AB311
- AB1157
- AB1206
- AG1
- B
- BB4
- BD792
- BL21
- BL21 (DE3)
- BM25.8
- C
- C-1a
- C-3000
- C25
- C41 (DE3)
- C43 (DE3)
- C600
- Cavalli Hfr
- CIE85
- DH1
- DH10 GOLD
- DH10B
- DH5
- DH5-alpha
- DP50
- DY145
- DY380
- E11
- EJ183
- EL250
- EMG2
- EPI 300
- EZ10
- FDA Seattle 1946
- Fusion-Blue
- H1443
- HF4714
- HB101
- HS(PFAMP)R
- Hfr3000
- Hfr3000 X74
- HMS174
- J52
- J53
- JC3272
- JC7661
- JC9387
- JF1504
- JF1508
- JF1509
- JJ055
- JM83
- JM101
- JM109
- K12
- KC8
- KA802
- KAM32
- KAM33
- KAM43
- LE450
- LE451
- LE452
- MB408
- MBX1928
- MC1061
- MC4100 (MuLac)
- MG1655
- MM294
- MS101
- NC-7
- Nissle 1917
- One Shot STBL3
- OP50
- P678
- PA309
- PK-5
- PMC103
- PR13
- Rri
- RV308
- S17-1λ -PIR
- SCS1
- SMR10
- SOLR
- SuperchargeEZ10
- SURE
- TOP10
- TG1
- U5/41
- W208
- W945
- W1485
- W3104
- W3110
- WA704
- WP2
- X1854
- X2160T
- X2541
- X2547T
- XL1-BLUE
- XL1-BLUE-MRF
- XLOLR
- Y10
- Y1090 (1090)
- YN2980
- W3110
- WG1
- WG439
- WG443
- WG445

The Office of Biohazard Containment and Safety (BCS) of the Canadian Food Inspection Agency (CFIA) only issues import permits for microorganisms that are pathogenic to animals, or parts of microorganisms that are pathogenic to animals. As the products listed above are not considered pathogenic to animals, the Office of BCS does not have any regulatory requirements for their importation.

Please note that other legislation may apply. You may wish to contact the Public Health Agency of Canada's (PHAC) Office of Laboratory Security at (613) 957-1779.

Note: Microorganisms pathogenic to animals and veterinary biologics require an import permit from the CFIA.

Sincerely,

Cinthia Labrie
Head, Animal Pathogen Importation Program
Office of Biohazard Containment & Safety

Canada

Adenovirus types 1, 2, 3, 4, 5 and 7 - Material Safety Data Sheets (MSDS)

MATERIAL SAFETY DATA SHEET - INFECTIOUS SUBSTANCES

SECTION I - INFECTIOUS AGENT

NAME: ***Adenovirus types 1, 2, 3, 4, 5 and 7***

SYNONYM OR CROSS REFERENCE: ARD, acute respiratory disease, pharyngoconjunctival fever

CHARACTERISTICS: *Adenoviridae*; non-enveloped, icosahedral virions, 70-90 nm diameter, doubled-stranded, linear DNA genome.

SECTION II - HEALTH HAZARD

PATHOGENICITY: Varies in clinical manifestation and severity; symptoms include fever, rhinitis, pharyngitis, tonsillitis, cough and conjunctivitis; common cause of nonstreptococcal exudative pharyngitis among children under 3 years; more severe diseases include laryngitis, croup, bronchiolitis, or severe pneumonia; a syndrome of pharyngitis and conjunctivitis (pharyngoconjunctival fever) is associated with adenovirus infection

EPIDEMIOLOGY: Worldwide; seasonal in temperate regions, with highest incidences in the fall, winter and early spring; in tropical areas, infections are common in the wet and colder weather; annual incidence is particularly high in children; adenovirus types 4 and 7 are common among military recruits (ARD)

HOST RANGE: Humans

INFECTIOUS DOSE: >150 plaque forming units when given intranasally

MODE OF TRANSMISSION: Directly by oral contact and droplet spread; indirectly by handkerchiefs, eating utensils and other articles freshly soiled with respiratory discharge of an infected person; outbreaks have been related to swimming pools; possible spread through the fecal-oral route

INCUBATION PERIOD: From 1-10 days

COMMUNICABILITY: Shortly prior to and for the duration of the active disease

SECTION III - DISSEMINATION

RESERVOIR: Humans

ZOONOSIS: None

VECTORS: None

SECTION IV - VIABILITY

DRUG SUSCEPTIBILITY: No specific antiviral available; cidofovir has shown promise in the treatment of adenoviral ocular infections.

SUSCEPTIBILITY TO DISINFECTANTS: Susceptible to 1% sodium hypochlorite, 2% glutaraldehyde, 0.25% sodium dodecyl sulfate

PHYSICAL INACTIVATION: Sensitive to heat>56°C; unusually stable to chemical or physical agents and adverse pH conditions

SURVIVAL OUTSIDE HOST: Resistance to chemical and physical agents allows for prolonged survival outside of the body. Adenovirus type 3 survived up to 10 days on paper under ambient conditions; adenovirus type 2 survived from 3-8 weeks on environmental surfaces at room temperature

SECTION V - MEDICAL

SURVEILLANCE: Monitor for symptoms; confirm by serological analysis

FIRST AID/TREATMENT: Mainly supportive therapy

IMMUNIZATION: Vaccine available for adenovirus types 4 and 7 (used for military recruits)

PROPHYLAXIS: None available

SECTION VI - LABORATORY HAZARDS

LABORATORY-ACQUIRED INFECTIONS: Ten cases documented up to 1988

SOURCES/SPECIMENS: Respiratory secretions

PRIMARY HAZARDS: Ingestion; droplet exposure of the mucous membrane

SPECIAL HAZARDS: Contact with feces from infected animals

SECTION VII - RECOMMENDED PRECAUTIONS

CONTAINMENT REQUIREMENTS: Biosafety level 2 practices and containment facilities for all activities involving the virus and potentially infectious body fluids or tissues

PROTECTIVE CLOTHING: Laboratory coat; gloves when skin contact with infectious materials is unavoidable

OTHER PRECAUTIONS: None

SECTION VIII - HANDLING INFORMATION

SPILLS: Allow aerosols to settle; wearing protective clothing gently cover the spill with absorbent paper towel and apply 1% sodium hypochlorite starting at the perimeter and working towards the centre; allow sufficient contact time (30 min) before clean up

DISPOSAL: Decontaminate all wastes before disposal; steam sterilization, incineration, chemical disinfection

STORAGE: In sealed containers that are appropriately labelled

SECTION IX - MISCELLANEOUS INFORMATION

Date prepared: November 1999

Prepared by: Office of Laboratory Security, PHAC

Although the information, opinions and recommendations contained in this Material Safety Data Sheet are compiled from sources believed to be reliable, we accept no responsibility for the accuracy, sufficiency, or reliability or for any loss or injury resulting from the use of the information. Newly discovered hazards are frequent and this information may not be completely up to date.

Date Modified: 2011-02-18

Adenovirus [2]

Virology: Adenoviruses are medium-sized (90-100 nm), nonenveloped icosohedral viruses containing double-stranded DNA. There are 49 immunologically distinct types (6 subgenera: A through F) that can cause human infections. Adenoviruses are unusually stable to chemical or physical agents and adverse pH conditions, allowing for prolonged survival outside of the body.

The adenovirus infection cycle can be clearly divided into two phases, which are separated by viral DNA replication. The first or "early" phase covers the entry of the virus into the host cell and the entry of the virus genome to the nucleus. The late genes are transcribed from the major late promoter. The "late" phase is involved in making gene products that are related to production and assembly of capsid proteins.

Adenoviral Genes	Function
Early genes (E): E1A, E1B, E2, E3, E4	Adenoviral gene transcription, replication, host immune suppression, inhibition of host cell apoptosis
Delayed early genes: IX, IVa2	Packaging
Major late Unit (L)	Assembly

Virus packaged by transfecting HEK 293 cells with adenoviral-based vectors are capable of infecting human cells. These viral supernatants could, depending on the gene insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*. For these reasons, due caution must be exercised in the production and handling of any recombinant adenovirus.

The probability of producing replication competent adenovirus (RCA), although low, Increases with each successive amplification. RCA is produced when adenoviral DNA recombines with E1-containing genomic DNA in HEK 293 cells. It is suggested that to use early amplification stocks when needed to produce additional quantities of adenovirus.

Clinical features: Adenoviruses most commonly cause respiratory illness; however, depending on the infecting serotype, they may also cause various other illnesses, such as gastroenteritis, conjunctivitis, cystitis, and rash illness. Symptoms of respiratory illness caused by adenovirus infection range from the common cold syndrome to pneumonia, croup, and bronchitis. Patients with compromised immune systems are especially susceptible to severe complications of adenovirus infection. Acute respiratory disease (ARD) can be caused by adenovirus infections..

Epidemiology: Although epidemiologic characteristics of the adenoviruses vary by type, all are transmitted by direct contact, fecal-oral transmission, and occasionally waterborne transmission. Some types are capable of establishing persistent asymptomatic infections in tonsils, adenoids, and intestines of infected hosts, and shedding can occur for months or years. Some adenoviruses (e.g., serotypes 1, 2, 5, and 6) have been shown to be endemic in parts of the world where they have been studied, and infection is usually acquired during childhood. Other types cause sporadic infection and occasional outbreaks; for example, epidemic keratoconjunctivitis is associated with adenovirus serotypes 8, 19, and 37. Epidemics of febrile disease with conjunctivitis are associated with waterborne transmission of some adenovirus types. ARD is most often associated with adenovirus types 4 and 7 in the United States. Enteric adenoviruses 40 and 41 cause gastroenteritis, usually in children. For some adenovirus serotypes, the clinical spectrum of disease associated with infection varies depending on the site of infection; for example, infection with adenovirus 7 acquired by inhalation is associated with severe lower

respiratory tract disease, whereas oral transmission of the virus typically causes no or mild disease.

Treatment: Most infections are mild and require no therapy or only symptomatic treatment. Because there is no virus-specific therapy, serious adenovirus illness can be managed only by treating symptoms and complications of the infection.

Laboratory hazards: Ingestion; droplet exposure of the mucous membrane

Laboratory Hazards	PPE
Exposure of mucus membrane (eyes, nose, mouth)	Use of safety goggles or full face shields. Use of appropriate face mask
Injection	Use of safety needles; NEVER re-cap needle or remove needle from syringe
Aerosole inhalation	Use of appropriate respiratory protection
Direct contact with skin	Gloves, lab coat, closed shoes

The above PPE are often required IN ADDITION to working in a certified Biosafety Cabinet.

Use with Animals: BSL-2 housing post injection/exposure of animals.

Adenovirus MSDS

Attachment 3:

1. HEK293 cell line- MSDS and Product Information.
2. SH-SY5Y cell line – MSDS and Product Information.

Cell Biology

ATCC® Number: **CRL-1573™** Order this Item Price:

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Designations:	293 [HEK-293]
Depositors:	FL Graham
Biosafety Level:	2 [CELLS CONTAIN ADENOVIRUS]
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens*
Morphology:	epithelial 
Source:	**Organ:** embryonic kidney
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Restrictions:	These cells are distributed for research purposes only. 293 cells, their products, or their derivatives may not be distributed to third parties.
Applications:	efficacy testing transfection host viruscide testing
Receptors:	vitronectin, expressed
Tumorigenic:	YES
DNA Profile (STR):	Amelogenin: X CSF1PO: 11,12 D13S317: 12,14 D16S539: 9,13 D5S818: 8,9 D7S820: 11,12 THO1: 7,9.3 TPOX: 11 vWA: 16,19
Cytogenetic Analysis:	This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells.
Age:	fetus

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Product Information Sheet

BioProducts

Cell, microbial and molecular genomics products for the life sciences

BioServices

Bio-materials management; basic repository to complex partnership-level services

BioStandards

Biological Reference Material and Consensus Standards for the life science community

Comments:

Although an earlier report suggested that the cells contained Adenovirus 5 DNA from both the right and left ends of the viral genome [RF32764], it is now clear that only left end sequences are present.
The line is excellent for titrating human adenoviruses.
The cells express an unusual cell surface receptor for vitronectin composed of the integrin beta-1 subunit and the vitronectin receptor alpha-v subunit.
The Ad5 insert was cloned and sequenced, and it was determined that a colinear seqment from nts 1 to 4344 is integrated into chromosome 19 (19q13.2).

Propagation:

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
The cell line does not adhere to the substrate when left at room temperature for any length of time, therefore, live cultures may be received with the cells detached.The cells will re-attach to the flask over a period of several days in culture at 37C.

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.An inoculum of 2 X 10 (3) to 6 X 10 (3) viable cells/cm2 is recommended.
6. Incubate cultures at 37°C.6. Subculture when cell concentration is between 6 and 7 X 10(4) cells/cm2.

Subcultivation Ratio: 1:6 to 1:10 weekly
Medium Renewal: Every 2 to 3 days

Preservation:

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2003
derivative: ATCC CRL-10852
derivative: ATCC CRL-12006
derivative: ATCC CRL-12007
derivative: ATCC CRL-12013
derivative: ATCC CRL-12479
derivative: ATCC CRL-2029
derivative: ATCC CRL-2368
purified DNA:ATCC CRL-1573D

Cell Line Designation: 293 (HEK293)
ATCC® Catalog No. CRL-1573™

Table of Contents:

Cell Line Description

Organism: *Homo sapiens* (human)
Tissue: kidney; transformed with adenovirus 5 DNA
Age: fetus
Morphology: epithelial
Growth properties: adherent
Doubling time: about 19 hours
Tumorigenic: tumors developed within 21 days at 100% frequency (5/5) in nude mice inoculated subcutaneously with 10(7) cells.
Receptors expressed: vitronectin
Virus susceptibility: human adenoviruses
DNA profile (STR analysis)

Amelogenin: X
CSF1PO: 11,12
D13S317: 12,14
D16S539: 9,13
D5S818: 8,9
D7S820: 11,12
TH01: 7,9.3
TPOX: 11
vWA: 16,19

Depositors: F.L. Graham
Comments: Although an earlier report suggested that the cells contained Adenovirus 5 DNA from both the right and left ends of the viral genome, it is now clear that only left end sequences are present. The line is excellent for titrating human adenoviruses.
The cell line does not adhere to the substrate when left at room temperature for any length of time, therefore, live cultures may be received with the cells detached. The cells will re-attach to the flask over a period of several days in culture at 37C.
The cells express an unusual cell surface receptor for vitronectin composed of the integrin beta-1 subunit and the vitronectin receptor alpha-v subunit.
The Ad5 insert was cloned and sequenced, and it was determined that a colinear seqment from nts 1 to 4344 is integrated into chromosome 19 (19q13.2).

Karyotype: This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %.
The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired.
There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells.
Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines.
Purified DNA: from this line is available as ATCC Catalog No. CRL-1573D™ (10 µg).

Biosafety Level: 2

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 2007. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Use Restrictions

These cells are distributed for research purposes only. 293 cells, their products, or their derivatives may not be distributed to third parties. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a **37°C** water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the

contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete growth medium and spin at approximately 125 xg for 5 to7 minutes.

4. Resuspend cell pellet with the recommended complete growth medium (see the specific batch information for the culture recommended dilution ratio) and dispense into a 25 cm^2 or a 75 cm^2 culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended ifusing the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.
The cell line does not adhere to the substrate when left at room temperature for any length of time, therefore, live cultures may be received with the cells detached. The cells will re-attach to the flask over a period of several days in culture at 37°C.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm^2 flasks; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Add 2.0 to 3.0 ml of 0.25% (w/v) Trypsin-0.53mM EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 10 minutes).

   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

3. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

4. Add appropriate aliquots of the cell suspension to new culture vessels. An inoculum of 2 X 10^3 to 6 X 10^3 viable cells/cm^2 is recommended.
   Subcultivation Ratio: 1:6 to 1:10 weekly

5. Incubate cultures at 37°C.

6. Subculture when cell concentration is between 6 and 7 X 10^4 cells/cm^2.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 13 in **Culture Of Animal Cells: A Manual Of Basic Technique** by R. Ian Freshney, 5th edition, published by Wiley-Liss, N.Y., 2005.

Medium Renewal

Two to three times weekly

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere.
ATCC tested fetal bovine serum is available as ATCC Catalog No. 30-2020 (500ml) and ATCC Catalog No. 30-2021 (100ml).

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO.
Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(additional references may be available in the catalog description at www.atcc.org)

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06/11

Attachment 2.3

Cell Biology

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Biosafety Level:	1
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Growth Properties:	mixed, adherent and suspension
Organism:	*Homo sapiens*
Morphology:	epithelial 
Source:	**Organ:** brain **Disease:** neuroblastoma **Derived from metastatic site:** bone marrow
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Restrictions:	NOTE: SH-SY5Y was deposited at the ATCC by June L. Biedler, Memorial Sloan-Kettering Cancer Center. SH-SY5Y is distributed for academic research purposes only. Memorial Sloan-Kettering releases the line subject to the following: 1.) SH-SY5Y or its products must not be distributed to third parties. Commercial interests are the exclusive property of Memorial Sloan-Kettering Cancer Center. 2.) Any proposed commercial use of SH-SY5Y including any use by a for-profit entity must first be negotiated with Director, Office of Industrial Affairs, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; phone (212) 639-6181; FAX (212) 717-3439.
Isolation:	**Isolation date:** 1970
Applications:	transfection host
Antigen Expression:	Blood Type A; Rh+
DNA Profile (STR):	Amelogenin: X CSF1PO: 11 D13S317: 11 D16S539: 8,13 D5S818: 12 D7S820: 7,10 THO1: 7,10 TPOX: 8,11 vWA: 14,18

Cytogenetic

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- Biological Reference Material and Consensus Standards for the life science community

Analysis:	modal number = 47; the cells possess a unique marker comprised of a chromosome 1 with a complex insertion of an additional copy of a 1q segment into the long arm, resulting in trisomy of 1q [22554]
Age:	4 years
Gender:	female
Comments:	SH-SY5Y cells have a reported saturation density greater than 1 X 10(6) cells/sq cm. They are reported to exhibit moderate levels of dopamine beta hydroxylase activity [PubMed ID: 29704].
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is a 1:1 mixture of ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003, and F12 Medium. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C
Subculturing:	**Protocol:** These cells grow as a mixture of floating and adherent cells. The cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites). Cells will aggregate, form clumps and float. Remove the medium with the floating cells, and recover the cells by centrifugation. Rinse the adherent cells with fresh 0.25% trypsin, 0.53 mM EDTA solution, add an additional 1 to 2 ml of trypsin solution, and let the culture sit at room temperature (or at 37C) until the cells detach. Add fresh medium, aspirate, combine with the floating cells recovered above and dispense into new flasks. **Subcultivation Ratio:** A subcultivation ratio of 1:20 to 1:50 is recommended **Medium Renewal:** Every 4 to 7 days
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Doubling Time:	48 hrs
Related Products:	recommended serum:ATCC 30-2020 parental cell line:ATCC HTB-11
References:	22554: Ross RA, et al. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J. Natl. Cancer Inst. 71: 741-749, 1983. PubMed: 6137586 23032: Biedler JL, et al. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res. 38: 3751-3757, 1978. PubMed: 29704

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Product Information Sheet for ATCC® CRL-2266™

Cell Line Designation: SH-SY5Y
ATCC® Catalog No. CRL-2266™

Table of Contents:

Cell Line Description

Organism: ***Homo sapiens*** (human)
Tissue: brain ;neuroblastoma; from metastatic site: bone marrow
Age: 4 years
Gender: female
Morphology: epithelial
Growth properties: mixed: suspension and adherent
DNA profile (STR analysis)
Amelogenin: X
CSF1PO: 11
D13S317: 11
D16S539: 8,13
D5S818: 12
D7S820: 7,10
TH01: 7,10
TPOX: 8,11
vWA: 14,18
AntigenExp: Blood Type A; Rh+
Depositors: .L. Biedler
Comments: SH-SY5Y is a thrice cloned (SK-N-SH -> SH-SY -> SH-SY5 -> SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH (see ATCC HTB-11) which was established in 1970 from a metastatic bone tumor.
The cells exhibit moderate levels of dopamine beta hydroxylase activity. SH-SY5Y cells have a reported saturation density greater than 1 X 10 exp6 cells/sq cm.
The cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites).Cells will aggregate, form clumps and float.
Karyology: modal number = 47; the cells possess a unique marker comprised of a chromosome 1 with a complex insertion of an additional copy of a 1q segment into the long arm, resulting in trisomy of 1q.
Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Use Restrictions

These cells are distributed for research purposes only. SH-SY5Y was deposited at the ATCC by June L. Biedler, Memorial Sloan-Kettering Cancer Center. SH-SY5Y is distributed for academic research purposes only. Memorial Sloan-Kettering releases the line subject to the following:
1. SH-SY5Y or its products must not be distributed to third parties. Commercial interests are the exclusive property of Memorial Sloan-Kettering Cancer Center.
2. Any proposed commercial use of SH-SY5Y including any use by a for-profit entity must first be negotiated with Director, Office of Industrial Affairs, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; phone (212) 639-6181; FAX (212) 717-3439.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium. and spin at approximately 125 xg for 5 to7 minutes.

4. Resuspend cell pellet with the recommended complete medium (see the specific batch information for the culture recommended dilution ratio). *It is important to*

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avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6). pH (7.0 to 7.6).

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

These cells grow as a mixture of floating and adherent cells.
Remove the medium with the floating cells, and recover the cells by centrifugation.
Rinse the adherent cells with fresh 0.25% trypsin, 0.53mM EDTA solution, add an additional 1 to 2 ml of trypsin-EDTA solution, and let the culture sit at room temperature (or at 37°C) until the cells detach.
Add fresh medium, aspirate, combine with the floating cells recovered above and dispense into new flasks.
Subcultivation ratio: 1:20 to 1:50

Medium Renewal

Every 4 to 7 days

Complete Growth Medium

The base medium for this cell line is a 1:1 mixture of ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003, and Ham's F12 Medium.

To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere.
ATCC tested fetal bovine serum is available as ATCC Catalog No. 30-2020 (500ml) and ATCC Catalog No. 30-2021 (100ml).

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO.
Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(additional references may be available in the catalog at www.atcc.org)

Ross RA et al. **Coordinate morphological and biochemical interconversion of human neuroblastoma cells.** J. Natl. Cancer Inst. 71: 741-749, 1983 PubMed: 84011813

Biedler JL et al. **Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones.** Cancer Res. 38: 3751-3757, 1978 PubMed: 79022839

Hay, R. J., Caputo, J. L., and Macy, M. L., Eds. (1992), **ATCC Quality Control Methods for Cell Lines**. 2nd edition, Published by ATCC.

Caputo, J. L., **Biosafety procedures in cell culture**. J. Tissue Culture Methods **11**:223-227, 1988.

Fleming, D.O., Richardson, J. H., Tulis, J.J. and Vesley, D., (1995) **Laboratory Safety: Principles and Practice**. Second edition, ASM press, Washington, DC.

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The viability of ATCC products is warranted for 30 days from the date of shipment. If you feel there is a problem with this product, contact Technical Services by phone at 800-638-6597 (U.S., Canada, and Puerto Rico) or 703-365-2700 (elsewhere) or by e-mail at tech@atcc.org.

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Attachment 4:

SN56 Cell line

– Brain Research 512(1990) 190-200. Development and characterization of clonal cell lines derived from septal cholinergic neurons.

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SN56/HN33 Murine Cell Lines

Applications

- Mouse model useful in studying the development of the central nervous system.
- Basic neurological research and drug testing.

Technical Summary

This novel cell line consists of neonatal mouse brain cells taken from the septum and fused with murine neuroblastoma to make an immortalized cell line. These cells exhibit phenotypic characteristics of differentiated septal neurons.

Contact Cliff Michaels, Licensing Associate ; 404-727-3890 ; ccmicha@emory.edu ;

Technology ID 97009

Inventor(s) Bruce Wainer;

Direct Link http://emoryott.technologypublisher.com/technology/4964

Brain Research, 512 (1990) 190–200
Elsevier

BRES 15302

Development and characterization of clonal cell lines derived from septal cholinergic neurons

David N. Hammond[1,2,5], Henry J. Lee[3], James H. Tonsgard[1,2,5] and Bruce H. Wainer[1,3,4,5]

Departments of [1]Pediatrics, [2]Neurology, [3]Pharmacological and Physiological Sciences, and [4]Pathology, and [5]the Joseph P. Kennedy Mental Retardation Center, The University of Chicago, Chicago, IL 60637 (U.S.A.)

(Accepted 15 August 1989)

Key words: Cholinergic; Hybrid cell line; Septum; Choline acetyltransferase; Neurofilament protein; Ultrastructure

Studies employing primary cells to determine the molecular basis of neuronal development and selective synaptogenesis in the central nervous system are limited by cellular heterogeneity. Clonal hybrid cell lines derived from a particular region of brain, which express differentiated characteristics typical of the cells of origin, offer a potentially powerful alternative approach. We previously demonstrated the feasibility of deriving such cell lines from septal cholinergic cells. We now delineate the methods employed, and describe the development of additional cholinergic cell lines expressing neuronal and cholinergic features from later developmental stages. One cell line has been studied in detail and found to form neurites, express choline acetyltransferase (ChAT) and neurofilament protein (NFP), and display typical neuronal ultrastructural characteristics, including puncta adherens, neuritic varicosities, vesicles, and growth cones.

INTRODUCTION

The mechanisms mediating neuronal development and selective synaptogenesis in the central nervous system remain poorly understood. Studies from this laboratory employing the septo-hippocampal system as a model have demonstrated that target hippocampal cells play an important role in the development of projecting cholinergic neurons from the septal region, and in the formation and maintenance of synapses[21–23]. Cell-surface recognition molecules may be important in this process, as may soluble trophic substances. For example, nerve growth factor (NGF) is produced by cholinoceptive target tissues, such as the hippocampus, and has been identified as a putative central cholinergic trophic factor[6,20,24,28,32,44,46]. The hippocampus may also produce soluble molecule(s) other than NGF which influence septal cholinergic neurons, possibly at different stages of development[4,37].

Primary cell culture has proven very useful in these studies. However, as an approach to studies of the molecular basis of neuronal development and selective synapse formation, primary cell culture is limited by the heterogeneity of cell preparations. Even distinct areas of the mammalian central nervous system contain neurons with different neurotransmitter phenotypes and synaptic connections, as well as non-neuronal cells. Advances in somatic cell fusion techniques provide an avenue to circumvent these problems through immortalization of neuronal cells by fusion with tumor cells. The somatic cell fusion technique has been used to only a limited extent in investigations of the central nervous system[35], and has not been previously applied to studies of specific regions of basal forebrain. We examined the possibility of generating clonal cell lines from cells of the septal region in order to study the molecular basis of cholinergic neuron development and selective synaptogenesis with greater resolution. We previously demonstrated the feasibility of deriving cell lines which express characteristics typical of septal cholinergic neurons[19]. We now provide a detailed description of the methods employed in the generation and characterization of septal hybrids, report the development of additional cholinergic cell lines expressing neuronal and cholinergic features, and describe the typical neuronal ultrastructural characteristics of these hybrid cells. Permanent cell lines such as these could greatly facilitate studies of central cholinergic neuron development, and of the trophic interactions which influence development and selective synaptogenesis.

Correspondence: D.N. Hammond, Departments of Pediatrics and Neurology, The University of Chicago, 5841 S. Maryland Avenue, Chicago, IL 60637, U.S.A.

MATERIALS AND METHODS

Polyethylene glycol-mediated fusion

Embryonic murine septal cells were fused with cells of the hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8)-deficient murine neuroblastoma line, N18TG2[18]. N18TG2 cells were cultured in Matalon's modified Eagle's medium (EMS) (Gibco), with 10% (v/v) fetal calf serum (FCS) and 0.1 mM 6-thioguanine (Sigma) to select against HPRT revertants, at 37 °C in 10% CO_2. Seven days before a fusion, the N18TG2 cells were subcultured in 6-thioguanine-free medium. Four days later, the cells were replated at a density of 15,000 cells per cm^2. On the day of fusion, the neuroblastoma cells were incubated for 10 min, at 37 °C, in 0.01% trypsin in calcium- and magnesium-free Tyrode's salt solution. After FCS-containing medium was added, the cells were harvested by mechanical agitation, centrifuged at 200 *g* for 10 min, and resuspended in medium plus 10% FCS. This cell suspension was plated on 60 mm Falcon tissue culture plates (Becton Dickinson) at a density of 2.5 $\times$ 10^6 cells per plate.

The septal region was dissected from C57BL/6 mouse embryos at day 14 of embryogenesis (E-14) for the first fusion, day 15 for the second fusion, and day 18 for the third. Septal cells were dissociated under sterile conditions, using methods previously described[21]. Under these conditions, viability of the cell suspensions ranged from 75 to 100%, as determined by Trypan blue dye exclusion. The primary septal cells were then washed once with serum-free medium, and resuspended (12.5 $\times$ 10^6 viable cells per ml) in serum-free medium containing phytohemagglutinin P (PHA-P) (Difco Lab) at a concentration of 100 μg per ml.

The fusion procedure described by Fournier was modified to fuse septal cells with N18TG2 cells[16]. The plated N18TG2 cells were washed gently with serum-free medium, after which 2.0 ml of the PHA-P septal cell suspension were added to each plate. The final ratio of primary brain cells to tumor cells was 10:1. The cells were then incubated for 15 min at 37 °C. Microscopic examination of the culture plate after incubation revealed that essentially all of the primary brain cells adhered to the N18TG2 monolayer. The PHA-P solution was then aspirated, and 1.5 ml polyethylene glycol (PEG) solution applied. The PEG solution was a 50/50 (w/w) solution of PEG, mol. wt. 1000 (Koch-Light 1000, Research International), in serum-free medium, at pH 7.6. After 1.0 min, the PEG solution was aspirated, and the plate rinsed quickly 3 times with serum-free medium. A volume of 3.5 ml of medium with 10% FCS was then added, and the plates incubated overnight at 37 °C in 10% CO_2. Control plates of primary septal cells or N18TG2 cells alone, treated in a similar manner, were also set up in parallel with the experimental plates.

On the following day, the medium was changed to one containing 10% FCS, hypoxanthine (100 μM), aminopterin (0.4 μM), and thymidine (16 μM) (HAT medium) which selects against unfused HPRT-deficient N18TG2 cells. The fusion products were harvested by mechanical agitation, and plated in HAT medium at 50,000 viable cells (as established by Trypan blue exclusion) per 35-mm tissue culture well (Falcon) for the E-14 fusion, 100,000 cells per 35-mm well for the E-15 fusion, and 100,000 cells per 6.4-mm microtiter well for the E-18 fusion. The medium used for the E-18 fusion also contained NGF, at 10 ng/ml, for the first 4 weeks in culture. Medium was changed every 3 days thereafter. Within 2–3 weeks, visible colonies were seen. Individual E-14 and E-15 colonies were isolated using cloning cylinders, and each was replated in a 16-mm well, in medium containing hypoxanthine, thymidine, and 10% FCS. E-18 fusion cells were cloned by limiting dilution. The colonies were expanded, and screened for the relevant characteristics. Cell lines were subsequently cultured in Dulbecco's modified Eagle medium (DMEM; Gibco), with 10% FCS.

Glucose phosphate isomerase (GPI; EC 5.3.1.9) isozyme analysis

Cells were grown in DMEM with 10% FCS to approx. 95% confluency on tissue culture plastic. They were rinsed with calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS; Gibco) at 37 °C, harvested by mechanical agitation, and centrifuged at 1,000 *g* for 5 min at 4 °C. The cells were then resuspended in ice-cold HBSS, and recentrifuged. The pellet was resuspended in an equal volume of extraction buffer (5 mM Tris, pH 7.5, 1 mM disodium EDTA, and 2.0% Triton X-100; Innovative Chemistry) for 30 min on ice, and then centrifuged at 2,000 *g* for 10 min at 4 °C. The supernatant was stored in liquid nitrogen. N18TG2 cell extracts prepared in the same fashion were employed as controls. A C57BL/6 adult brain extract, prepared as follows, also served as a control. The brain was removed, cut in half, quick frozen in liquid nitrogen, and stored at –80 °C. Half of the brain was then thawed, rinsed 3 times in ice-cold HBSS, and homogenized in a Ten Broeck tissue grinder in 360 μl of ice-cold extraction buffer containing 25 μg/ml deoxyribonuclease I (3381 U per mg dry wt., Worthington Biochemical Corporation). The homogenate was centrifuged at 2,000 *g* for 10 min at 4 °C, and the supernatant stored in liquid nitrogen. Electrophoresis was performed at 4 °C for 50 min, at a constant voltage of 200 V. Electrophoresis gels contained 1% agarose and 5% sucrose in an aminomethylpropanol buffer at pH 8.9; the electrode buffer was sodium alkaline barbital buffer (50 mM, pH 8.6) (both from Innovative Chemistry). Bands for GPI activity were revealed by staining the gel at 37 °C using commercially available GPI substrate (Innovative Chemistry).

Immunocytochemistry in monolayer culture

Cell lines were analyzed immunocytochemically for type-specific antigens in monolayer culture. A suspension of 1000–1500 cells was added to each well of an 8-chamber Lab-Tek glass slide (Miles Scientific) which had been coated with poly-D-lysine (Sigma; 100 μg/ml in 15 mM borate buffer, pH 8.3) for 12–24 h. The cells were cultured in medium with 10% FCS, and stained after 3–6 days in culture. Cells were fixed for 30 min in a solution of 0.5% paraformaldehyde, 0.001% glutaraldehyde, and 0.1% Triton X-100 in 50 mM phosphate buffer at pH 7.4. Monoclonal antibodies 4.3F9 (also referred to as TA51) which reacts with the carboxyl-terminal domains of phosphorylated neurofilament subunits NF150 and NF200[7,30,47], AB8 directed against ChAT[31,42,49], and 2.2B10.6 which reacts with glial fibrillary acidic protein (GFAP)[48] were employed. Immunostaining was performed using the peroxidase-antiperoxidase method of Sternberger as described[42]. Non-immune rat immunoglobulin G was employed as a control.

ChAT activity

Cell lines were grown in medium containing 10% FCS on Falcon tissue culture plates, and harvested by mechanical agitation. Cells were sonicated in citrate-phosphate buffer (50 μM citrate, 10 μM phosphate; pH 7.4), and ChAT activity measured as described by Fonnum[14]. ChAT specific activity was expressed as picomoles acetylcholine formed per minute per milligram protein (pmol/min/mg protein). Protein was measured by the method of Bradford[5].

Effect of NGF on ChAT activity and on process formation

SN cells were plated on tissue culture plastic (Falcon 100-mm plates) in medium containing 10% FCS and 100 ng/ml of NGF. The medium was replaced after 3 days with fresh medium containing NGF. The cells were harvested for ChAT activity assay after 6 days in culture. While in culture, the cells were examined daily by phase-contrast microscopy for qualitative changes in process formation.

Electron microscopy

SN17 cells were grown in medium containing 10% FCS. The cells were fixed with 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 30 min. The cells were postfixed with 1% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.4), dehydrated, and embedded in Durcupan resin. Ultrathin sections were then cut parallel to the cell layer and placed on Formvar-coated slot grids. The sections were stained with uranyl acetate and lead citrate, and examined under a Philips 201 electron microscope.

RESULTS

Three fusions of septal cells and N18TG2 cells were performed, employing primary cells from different embryonic ages which spanned the period of active septal cholinergic cell neurogenesis in the rodent[43]. The first and second fusions employed septal cells from embryos at day 14 and 15, respectively, the approximate time of peak final mitosis for septal cholinergic cells. The third fusion employed cells from septa at E-18, after the completion of neurogenesis[43]. Nine cell lines from the E-14 fusion (SN1–9), 8 lines from the E-15 fusion (SN10–17), and 17 lines from the E-18 fusion (SN18–34) were isolated and expanded in culture. Primary septal cells or N18TG2 cells alone, treated in a similar manner, produced no viable colonies.

Neurochemical studies

We elected to screen the viable cell lines from the 3 fusions for the expression of the definitive cholinergic marker, ChAT, as measured by the specific activity of the enzyme. The results of the E-15 and E-18 fusion screenings are depicted in Fig. 1. Most of the cell lines demonstrated ChAT activities similar to that of the N18TG2 parent cell type (22 pmol/min/mg protein). However, SN17 and a number of lines from the E-18 fusion (including SN18, SN30, and SN32), as well as two E-14 lines reported previously (SN5 and SN6)[19], showed significantly greater ChAT specific activities, ranging up to 237 pmol/min/mg protein. We also evaluated the effects of NGF on the ChAT activities of a series of SN lines, and found that the addition of NGF did not significantly increase ChAT activity under the routine culture conditions described above. SN5, SN6, and SN17 were subsequently studied in more detail.

SN5 and SN6 cells were initially karyotyped and found to have greater numbers of chromosomes than either parent cell type[19]. Karyotyping thus demonstrated that the cells were hybrids, but did not establish the identity of the parent cells. In order to determine the identity of the parent cells, SN5 and SN6 were subsequently studied by GPI isozyme analysis. It had been shown previously that A strain mice (from which N18TG2 cells were derived) and C57BL/6 mice (source of the primary septal cells) expressed electrophoretically distinct variants of GPI[18]. Furthermore, cells from GPI heterozygotes had been found to express the variant of each parent as well as an intermediate form[12,13]. SN5 and SN6 were studied and were found to express the GPI genes of both C57BL/6 and N18TG2 cells[19]. Since GPI analysis provided a more direct assessment of the cells of origin than karyotyping, in addition to demonstrating the hybrid nature of the lines, it was employed to evaluate SN17 cells. Extracts of N18TG2 neuroblastoma cells and of C57BL/6 mouse brain cells were used as electrophoretic standards. As shown in Fig. 2, GPI from N18TG2 cells and from C57BL/6 brain had different electrophoretic mobilities. SN17 cells expressed the electrophoretic variants of both parental strains, as well as an intermediate form, which did not appreciably migrate under

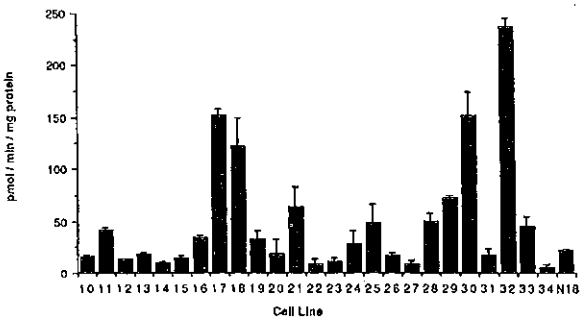

Fig. 1. ChAT activities of SN 10–34 and of N18TG2. ChAT activities of cell lines generated by fusion of septal and N18TG2 cells and of parent N18TG2 (N18) cells are displayed, as measured in pmoles of acetylcholine formed per min per mg protein. Lines SN10–17 were derived from the fusion of E-15 primary septal cells with N18TG2 cells and lines SN18–34 were derived using E-18 septal cells. ChAT activities of lines SN1–9, generated by a fusion of E-14 septal cells with N18TG2 cells, have been previously reported[19]. The values shown are means ± S.E.M (n = 3).

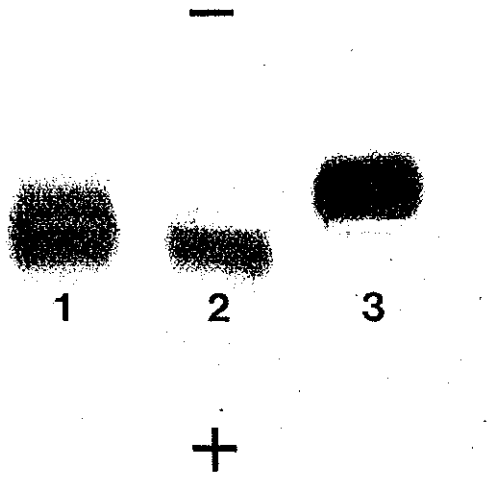

Fig. 2. Gel electrophoresis of SN17 GPI. Under the conditions described (see Experimental procedures), N18TG2 cell GPI migrates towards the anode (lane 2) and C57BL/6 GPI towards the cathode (lane 3). SN17 cells (lane 1) express the electrophoretic variants of both parental strains, as well as an intermediate form, which does not appreciably migrate under these conditions. SN17 cells are thus hybrids capable of the expression of genes from each of the parent cell types.

these conditions. This demonstrated that these cells were hybrids derived from the fusion of C57BL/6 and N18TG2 cells. The analysis also indicated that the SN cells were indeed capable of expressing genes from each of the parent cell types.

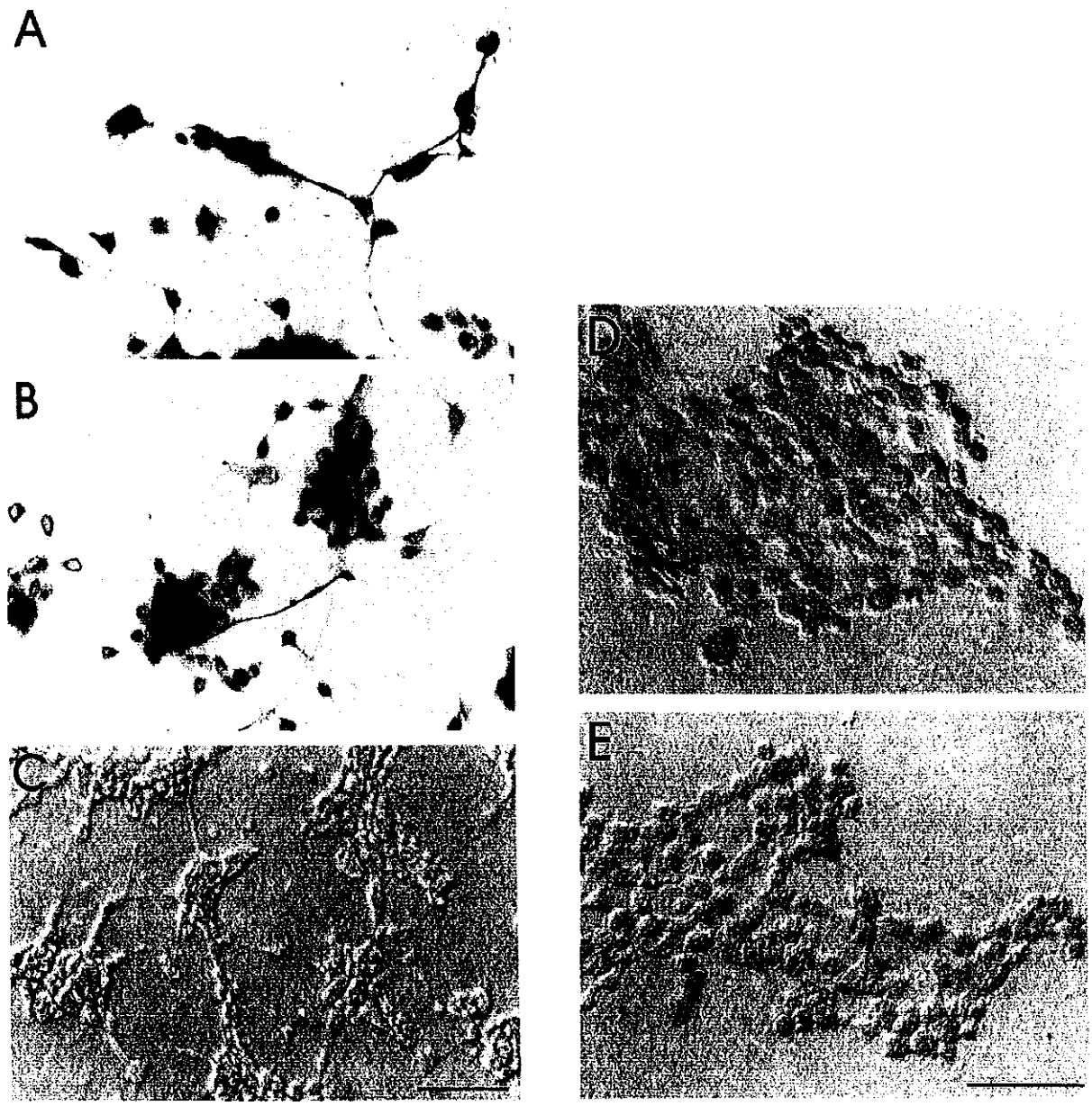

Fig. 3. A–C: immunocytochemistry of SN17 cells in monolayer culture. SN17 cells were grown in medium with 10% FCS, and stained using the immunoperoxidase method. Bar = 100 μm. (A) SN17 cells visualized immunocytochemically with monoclonal antibody AB8 against ChAT. There is staining of perikarya and processes, demonstrating the presence of ChAT immunoreactivity. (B) SN17 cells stained immunocytochemically using monoclonal antibody 4.3F9 directed against NFP. NFP-immunoreactivity is noted in perikarya and neuritic processes. (C) SN17 cells stained with non-immune rat immunoglobulin, as a control. Note the absence of significant staining. Differential interference contrast optics allows the visualization of somata and prominent processes despite the absence of immunocytochemical reaction product. The appearance of the cells was similar when they were stained with monoclonal antibody 2.2B10.66 directed against GFAP (not shown). D+E: N18TG2 cells stained (D) with AB8, monoclonal antibody against ChAT, and (E) with monoclonal antibody 4.3F9 directed against NFP. The cells were processed in the same way as the SN cells shown in Fig. 3A and B, using the immunoperoxidase method. There is no appreciable staining with either monoclonal antibody. Differential interference contrast optics allows the visualization of N18TG2 cells despite the absence of immunocytochemical reaction product. Unlike SN cells, N18TG2 cells do not exhibit the AB8 antigen or the 4.3F9 antigen; nor do they form prominent processes. Bar = 50 μm.

Morphology/immunocytochemistry

SN17 cells, as well as SN5 and SN6 cells, were found to display some morphologic characteristics typical of neurons in monolayer culture. When grown in medium containing 10% FCS, and viewed by phase-contrast microscopy, the cells were refractile, with somata which were round to oval in shape. In addition, the cells displayed prominent neuritic processes (see Fig. 3), some greater than 200 μm in length. N18TG2 cells did not demonstrate significant process formation under the same conditions in monolayer culture (Fig. 3). The effect of NGF on SN cell process formation was also evaluated by phase-contrast microscopy. The addition of NGF did not affect process formation under the routine culture conditions described above.

SN5 and SN6 had previously been shown to express neurofilament protein (NFP) and ChAT antigens[19]. We also analyzed SN17 cells immunocytochemically for cell type-specific antigens, using monoclonal antibodies against ChAT, NFP, and GFAP. AB8, a monoclonal

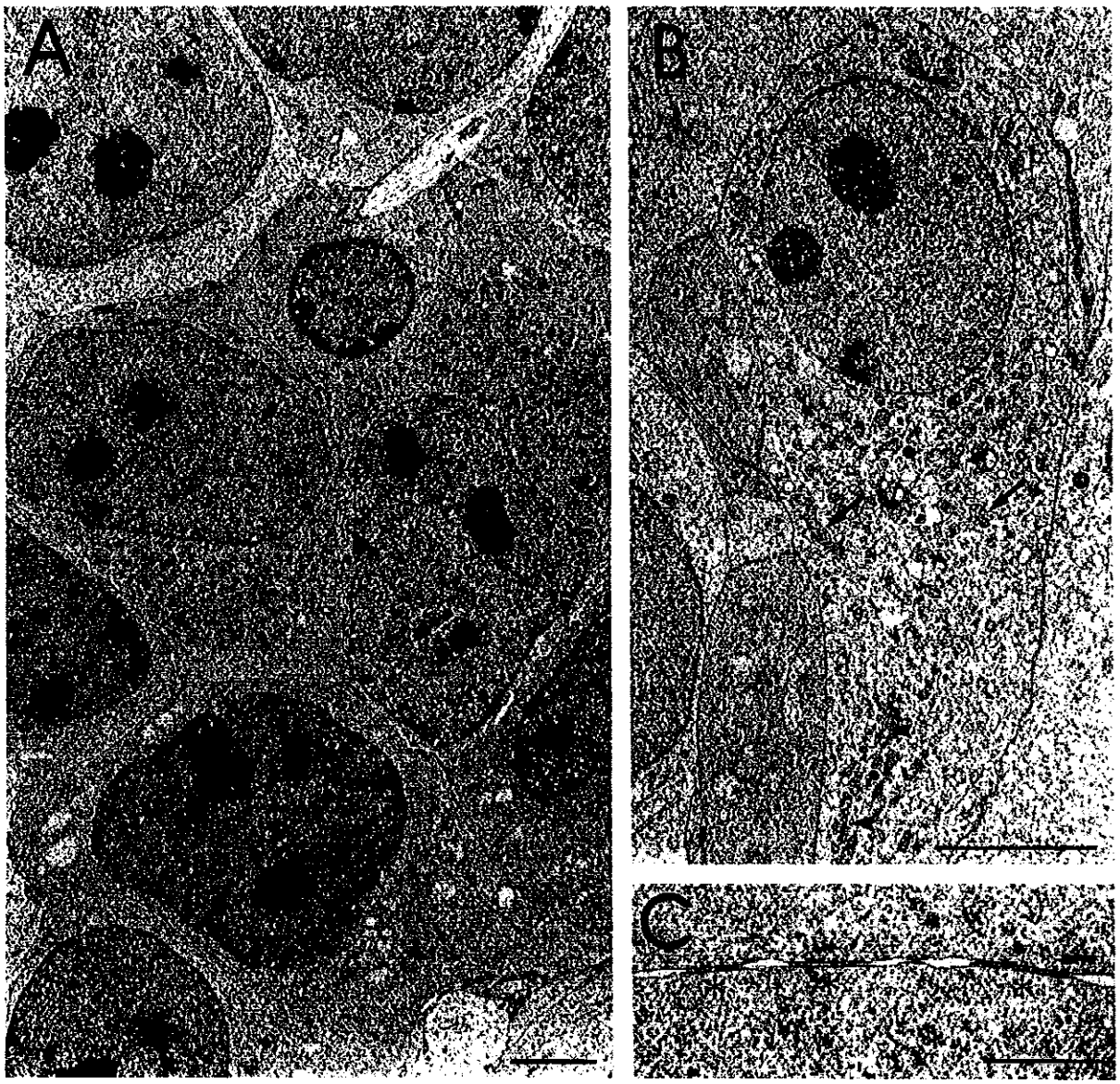

Fig. 4. Transmission electron micrographs of the somata of confluent SN17 cells. In (A), and, at higher power, in (B), the somata can be seen to contain numerous mitochondria (small arrows) and the rough endoplasmic reticulum is prominent (arrowheads). Bars = 5μm. (C) SN 17 cells display membrane specializations which are readily seen at higher power (asterisks). The intercellular cleft is maintained. There are no vesicles adjacent to the junctional complex. Bar = 0.5 μm.

antibody against ChAT, has been used extensively to immunohistochemically identify mammalian central cholinergic neurons, including those of the septal region[42,49]. AB8 reacted with the cell bodies and neurites of SN17, as shown immunoctyochemically (Fig. 3A). AB8 did not label N18TG2 cells under the same conditions (Fig. 3D). The SN17 cell line also stained with 4.3F9 (Fig. 3B), a monoclonal antibody against NFP, while N18TG2 cells did not (Fig. 3E). While every SN17 cell stained with AB8 and with 4.3F9, there was some variability in the intensity of staining (Fig. 3). Cells whose soma and/or neuritic processes were in close contact with other cells tended to stain more intensely than those that were more isolated on the plate. SN17 cells did not, however, display the astrocyte-specific intermediate filament protein, GFAP, as detected by immunostaining with monoclonal antibody 2.2B10.6. Nor did the ChAT-positive lines stain when, as a control, non-immune rat immunoglobulin G was used in place of a monoclonal antibody (Fig. 3C).

The ultrastructural characteristics of SN17 somata, neuritic processes, and growth cones were also studied. Dense mitochondria and rough endoplasmic reticulum were present in the SN17 cell bodies, as seen in a field of confluent cells in Fig. 4A and B. Junctional complexes, with the maintenance of an intercellular cleft, were apparent at higher power (Fig. 4C). In no case were vesicles seen to be associated with these membrane specializations. Large and small dense core vesicles were also seen in some of the cell bodies (Fig. 5).

SN17 cell processes were also examined under the electron microscope. The processes contained interme-

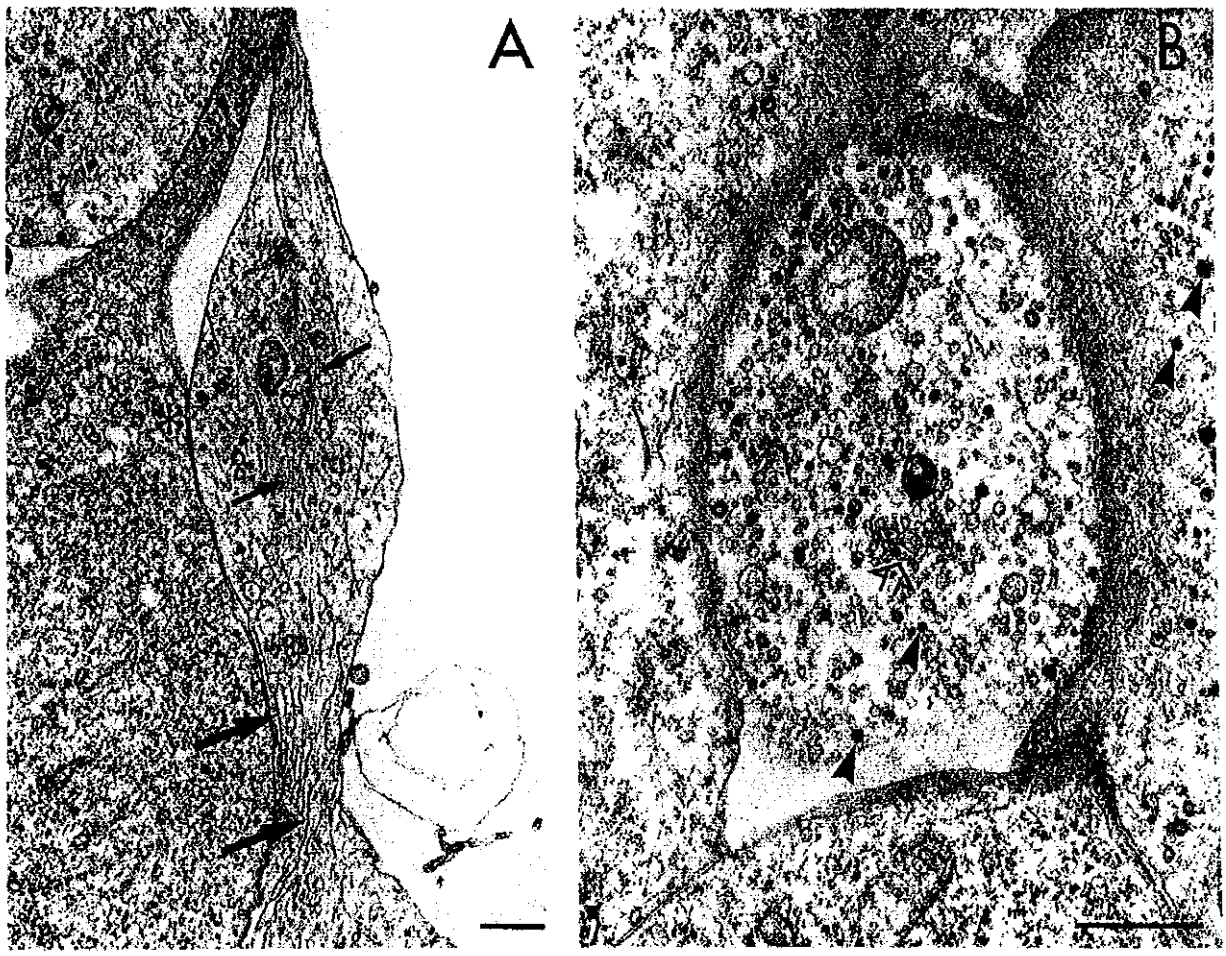

Fig. 5. Transmission electron micrographs of SN17 neuritic processes. The neurites display varicosities. A prominent varicosity is seen in longitudinal section in (A). Intermediate filaments (small arrows) and microtubules (large arrows) are present. Some varicosities display membrane specializations (asterisk). Bar = 1 μm. A varicosity of a different neurite is seen in cross-section in (B). Both dense core (dark arrowheads) and clear vesicles (open arrowhead) are present in the varicosities, although they do not tend to cluster in groups near the membrane. The cell body adjacent to the varicosity in (B) also contains dense core vesicles (dark arrowheads). Bar = 1 μm.

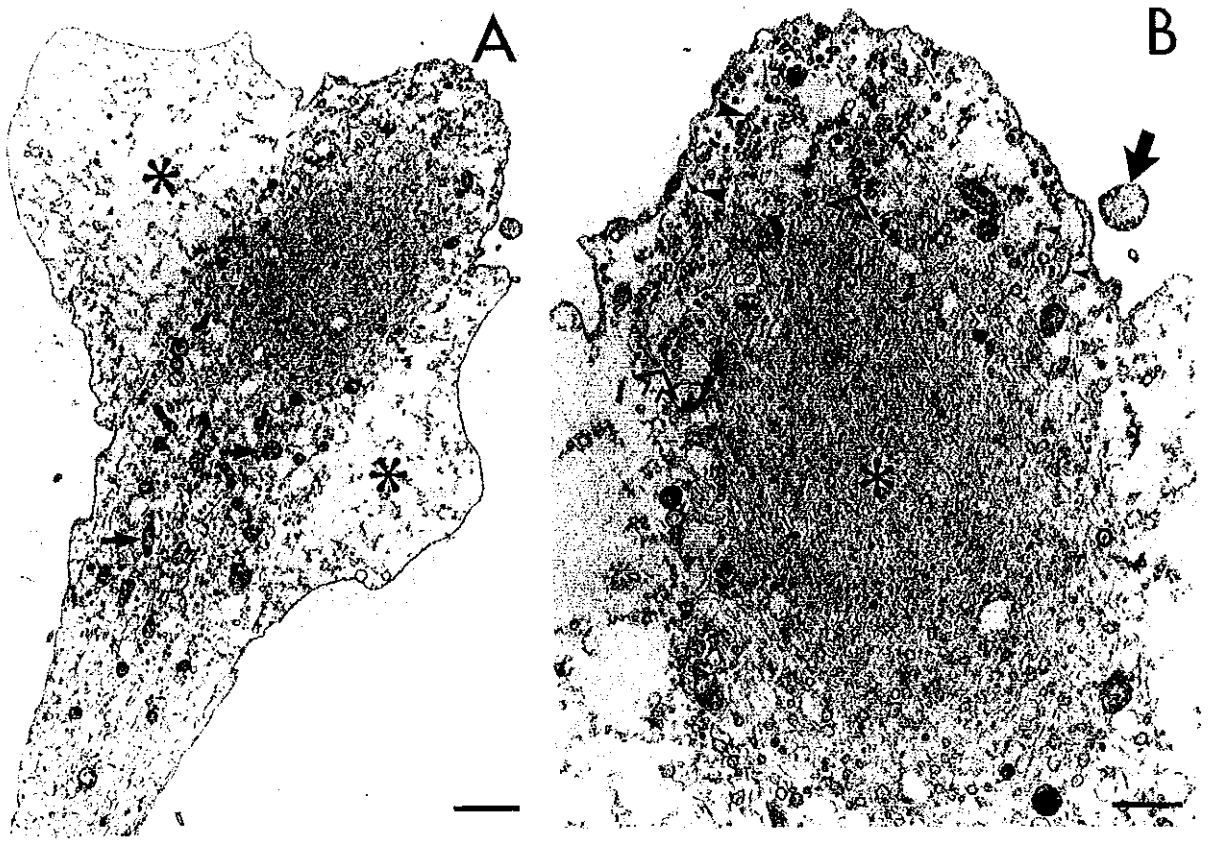

Fig. 6. Transmission electron micrographs of the distal region of a neurite of an SN17 cell. (A) The region is expanded, and displays flattened processes (lamellipodia), typical of neuronal growth cones (asterisk). Numerous mitochondria are seen (small arrows). Bar = 2 μm. (B) A higher power view of the same region. Numerous clear and dense core vesicles are present (open and closed large arrowheads) and a complex meshwork of microfilaments can be seen extending towards the periphery of the growth cone (asterisk). Polyribosomes are also seen (small arrowheads). Associated small processes, which may be filopodia in cross-section, are also present (large arrow). As is the case in normal neurons, these filopodia-like processes contain predominantly microfilaments. Bar = 1 μm.

diate filaments and microtubules, and frequently exhibited prominent varicosities (Fig. 5A and Fig. 5B). Some neuritic varicosities displayed membrane specializations (Fig. 5A). The varicosities also contained both dense core and clear vesicles, similar in appearance to synaptic vesicles. The vesicles, did not, however, tend to cluster near the cell membrane.

SN17 cells also displayed neurites with expanded regions typical of neuronal growth cones (Fig. 6). Flat processes similar to the lamellipodia of normal axonal growth cones extended from these regions. At higher power (Fig. 6B), large numbers of dense core vesicles, clear vesicles, and polyribosomes were apparent. Mitochondria were also present in these expanded regions. A meshwork of microfilaments extended peripherally, as is typical of normal neuronal growth cones. Associated small processes, which may be filopodia in cross-section, were also present. Like normal filopodia, these small processes were made up mainly of bundles of microfilaments.

DISCUSSION

We chose to develop cholinergic cell lines using somatic-cell hybridization techniques because this approach allows the immortalization of neurons which have undergone terminal mitosis. Oncogene transduction, on the other hand, while a valuable method of neural cell immortalization, requires that the target cell undergo at least one round of DNA synthesis for stable integration of the exogenous DNA to occur[8]. We examined the feasibility of using the somatic-cell hybridization approach to derive cell lines at approx. the time of peak final mitosis for septal cholinergic cells in the rodent (E-14 and E-15), as well as shortly after the completion of neurogenesis (E-18)[43]. In each case, hybrid cell lines expressing characteristics typical of septal cells were obtained.

Somatic-cell hybridization technical parameters were adjusted to optimize cell fusion and the expression of cholinergic neuronal traits. We found that the use of

phytohemagglutinin-P and the duration of exposure to PEG affect the efficiency of fusion. Primary septal cells do not adhere well to N18TG2 cells when standard monolayer fusion techniques are used. The use of PHA-P promotes the adherence of essentially all primary cells to the N18TG2 monolayer, and dramatically improves the efficiency of fusion. Duration of exposure to PEG is also critical. Exposure to PEG for longer than 1.0 min under these conditions results in marked cell death, probably due to the cytotoxicity of PEG itself. The efficiency of fusion declines with shorter exposure times. N18TG2 cells were chosen as the fusion partner for septal cells because the former are embryologically related to neurons[1], and thus likely to permit the expression of neuron-specific traits[25]. At the same time, N18TG2 is deficient in ChAT, permitting the use of this specific cholinergic marker in screening colonies. The murine origin of both the neuroblastoma line and the normal parent cells minimizes chromosomal loss which often occurs in xenogeneic fusions[26,34].

Using the somatic cell fusion approach, a series of cell lines has been developed which are hybrids of septal cells and N18TG2 cells. The hybrid nature of these cell lines is demonstrated by their growth in HAT medium and the presence of both parental GPI isozymes. The SN cell lines survive and grow in HAT medium, while neither parent cell type alone, on control plates, survives. Unfused HPRT-deficient N18TG2 do not survive because of the presence of aminopterin in the medium. Thus, the growth of SN cells indicates expression of the HPRT gene necessarily contributed by primary septal cells. Unfused primary septal cells do not survive for extended periods of time under these conditions. GPI isozyme analysis confirms the presence in SN17, SN5, and SN6 of genes from each parent cell type, and in addition indicates that these genes are actively expressed.

A number of the hybrid cell lines derived from the septal region, including SN5, SN6, and SN17, display neuronal and cholinergic features characteristic of septal cholinergic neurons, but not demonstrated by N18TG2 cells. When grown in the absence of any specific inducing agents, SN17, as well as SN5 and SN6, display prominent neurites which stain with 4.3F9, a monoclonal antibody which reacts with the carboxyl-terminal domains of the NF150 and NF200 subunits in the phosphorylated state[7]. The astrocytic intermediate filament protein, GFAP, as detected by monoclonal antibody 2.2B10, is not present. The cell lines also express the definitive cholinergic marker, ChAT. ChAT activity is clearly demonstrated by the enzyme activity assay of Fonnum, which has been shown to be highly specific for ChAT[14]. Additionally, the lines display ChAT immunoreactivity as detected by the monoclonal antibody AB8, which has been extensively characterized and found to be specific for ChAT[31,42,49]. While all SN17 cells express NFP and ChAT antigens as demonstrated immuncytochemically, those cells whose soma and/or neuritic processes are in close proximity to other cells tend to stain most intensely. Thus the degree of NFP and ChAT expression, at least as detected immunocytochemically, may be influenced by the microenvironment of the cell, including cell–cell contacts. Cell cycle stage may also influence the degree of expression of particular markers. It will be of interest in the future to evaluate the cell lines for the expression of other traits associated with cholinergic cells, such as high-affinity choline uptake and acetylcholine release[3].

SN17 cells also exhibit ultrastructural characteristics typical of normal neurons, including puncta adherens, growth cones, and varicosities. Puncta adherens, composed of junctional complexes with the maintenance of an intercellular cleft, are seen in normal neural tissue between adjacent neuronal somata[45], and between somata and dendrites[17], dendrites and axons[40] and axon terminals and initial segments of axons[39]. SN17 neurite terminals have the typical appearance of neuronal growth cones[27]. These expanded regions contain large numbers of vesicles (clear and dense core), mitochondria, and clustered ribosomes, with an extensive central network of microfilaments extending towards the periphery. Flattened sheet-like processes termed lamellipodia, and long, thin projections called filopodia are also seen.

SN17 cells also display neuritic swellings, or varicosities, which are typical of primary septal cholinergic cells in culture[21,22], and which contain clear and dense core vesicles. Clear vesicles are typically seen in the axon terminals of septal cholinergic neurons in situ[50], where they probably represent storage sites for acetylcholine. The function of large dense core vesicles (80–90 nm in diameter) is unclear[41]. They have been described in various regions of the central nervous system, sometimes in the company of smaller clear vesicles[9]. Large as well as small (40–60 nm diameter) dense core and clear vesicles are seen in neuronal growth cones[27]. Some SN17 neuritic varicosities also display membrane specializations. However, cultured alone, the SN17 cells do not display fully mature synapses with vesicles clustered near the cell membrane.

The effects of NGF on process formation and on ChAT activity of SN cell lines were also studied. None of the cell lines tested show significantly increased ChAT activity, or enhanced process formation, when cultured in the presence of NGF. Cell lines expressing low basal ChAT activity under control conditions may not respond because they have been derived from non-cholinergic septal cells, and thus lack the cholinergic phenotype. ChAT-positive SN cell lines may constitutively express maximal

enzyme activity under control culture conditions in the presence of 10% FCS, making any NGF effect undetectable.

Alternatively, SN lines may not respond to NGF, or display mature synapses (see above), because their phenotypic repertoire is limited to that of embryonic septal cells. Studies of hybrid cell lines derived from immature B lymphocytes by somatic-cell hybridization techniques demonstrate that these hybrid cells cannot be stimulated to express a fully differentiated phenotype, despite treatment with a variety of cytokines and differentiating reagents[38]. Similarly, embryonic septal cell lines may not respond fully to NGF or display mature synapses (see above) because their phenotype is 'locked in' to that of the embryonic stage from which they are derived. When, precisely, septal neurons in situ begin to respond to NGF is not clear, but certainly the principal response occurs in the postnatal period: hippocampal NGF is barely detectable at birth, and does not rise to a peak until postnatal day 21, this rise preceding, and presumably stimulating, the increase in septal ChAT activity which does not peak in situ until approx. 3 weeks postnatal[28]. SN17 cells display ultrastructural traits similar to those expected for embryonic septal cells in situ. SN17 does not, however, display fully mature synapses. This may be due to a requirement for target cells, or may occur because the ultrastructural repertoire of SN17 cells is limited to that of the embryonic stage from which they are derived. Mature synapses between septal cells and target hippocampal cells probably do not begin to occur in situ until the early postnatal period: the vast majority of mature synapses in the hippocampus are formed between approx. 1.5 and 4 weeks postnatal, with fewer than 1% of the adult number seen on postnatal day 4[11]. Since the SN cells appear to express characteristics typical of the embryonic septal cholinergic cells from which they are derived, they may prove especially useful in examining the regulation of immature septal neurons. For example, embryonic SN cell lines may respond to non-NGF trophic factors operative early in embryonic development, and thus facilitate studies of their effects on septal cholinergic neuron development.

It is probable, for a number of reasons, that the cholinergic and neuronal characteristics of the SN hybrid cells reflect the expression of C57BL/6 septal cell genes. SN cells are clearly capable of C57BL/6 gene expression, as demonstrated by GPI isozyme analysis, and the cholinergic and neuronal traits they constitutively express are demonstrated by septal cells, but not by N18TG2 cells. Furthermore, N18TG2 fusion is not sufficient in and of itself to induce the expression of septal cholinergic traits, such as ChAT: some septal X N18TG2 lines do not express activities greater than the parent neurotumor cells (see Fig. 1). Additionally, in other studies, we have fused N18TG2 cells with cells from hippocampus[29] and from rostral mesencephalic tegmentum[10], essentially non-cholinergic regions, and have found that these hybrid lines do not express ChAT. Nonetheless, new synthesis of a protein coded by a previously silent gene has been described in somatic cell hybrids[33]. Indeed, this phenomenon has provided insight into the regulation of cell differentiation[15]. Thus, the possibility that the cholinergic and neuronal characteristics observed in SN17, SN5 and SN6 cells result from selective activation of N18TG2 genes cannot be completely excluded.

While a few hybrid cell lines have been derived from the central nervous system previously to those described here, we know of none which specifically involve cells from the cholinergic groups of the basal forebrain. Chinese hamster brain cells have been fused with neurotumor cells[35], and have been useful in various studies, including those on the roles of cyclic AMP in modulating synapse formation and in receptor-mediated effects[36]. However, the septal lines described here are the first cell lines derived from a specific region of the basal forebrain. These hybrid cell lines are especially interesting because they express properties characteristic of a particular cell type of that region, the cholinergic neurons. Cell lines such as these, derived from a particular region of brain, which express neurochemical and ultrastructural characteristics typical of the cells of origin, provide significant advantages over primary cultures[2], and may greatly facilitate studies of neuronal development, and of the trophic interactions which influence development and selective synaptogenesis.

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