- Explore a product's biodistribution characteristics using various imaging technologies

Whatever the goal of the study, exploratory IND studies can help identify, early in the process, promising candidates for continued development and eliminate those lacking promise. As a result, exploratory IND studies may help reduce the number of human subjects and resources, including the amount of candidate product, needed to identify promising drugs. The studies discussed in this guidance involve dosing a limited number of subjects with a limited range of doses for a limited period of time.

Existing regulations provide more flexibility with regard to the preclinical testing requirements for exploratory IND studies than for traditional IND studies. However, sponsors submitting the kinds of studies described in this guidance have not always taken full advantage of that flexibility. Sponsors often provide more supporting information in their INDs than is required by the regulations. Because exploratory IND studies involve administering either sub-pharmacologic doses of a product, or doses expected to produce a pharmacologic, but not a toxic, effect, the potential risk to human subjects is less than for a traditional phase 1 study that, for example, seeks to establish a maximally tolerated dose. ***Because exploratory IND studies present fewer potential risks than do traditional phase 1 studies that look for dose-limiting toxicities, such limited exploratory IND investigations in humans can be initiated with less, or different, preclinical support than is required for traditional IND studies.***[6]

The Agency expects that this early phase 1 exploratory IND approach will apply to a number of different study paradigms. Although his guidance explores several potential applications, many others can be proposed. The Agency believes that, consistent with its Critical Path Initiative, clarifying Agency thinking about how much and what kind of testing is needed to support early studies in humans will facilitate the entry of new products into clinical testing and speed product development.

Although exploratory IND studies may be used during development of products intended for any indication, it is particularly important for manufacturers to consider this approach when developing products to treat serious diseases. Because the approach can help identify promising candidates more quickly and precisely, exploratory IND studies could become an important part of the armamentarium when developing drug and biological products to treat a serious or life-threatening illness. The Agency has previously articulated its commitment to ensuring that appropriate flexibility is applied when patients with a serious disease and no satisfactory alternative therapies are enrolled in a trial with therapeutic intent.[7]

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in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body.)

[6] Generally, these types of studies would not be carried out in pediatric patients or in pregnant or lactating women.

[7] Subpart H Accelerated Approval of New Drugs for Serious or Life-Threatening Illnesses. See also FDA guidance for industry *Fast Track Drug Development Programs — Designation, Development, and Application Review.*

III. CONTENT OF IND SUBMISSIONS

To begin any kind of testing in humans, applicants must submit an IND application to the Agency with certain types of information (see 21 CFR 312.23 IND Content and Format). The primary purpose of the IND submission is to ensure that subjects will not face undue risk of harm. The major information that must be submitted includes:

- Information on a clinical development plan
- Chemistry, manufacturing, and controls information
- Pharmacology and toxicology information
- Previous human experience with the investigational candidate or related compounds, if there is any

The following sections discuss the first three in more detail. Because the exploratory IND studies addressed by this guidance will be first in human studies, previous human experience is not pertinent and will not be discussed. The common theme throughout is that, depending on the study, the informational requirements for exploratory IND studies are more flexible than for traditional IND studies.

A. Clinical Information

1. Introductory statement and general investigational plan

A traditional IND application describes the rationale for the proposed clinical trial program and discusses the potential outcome of the clinical investigation. The exploratory IND studies discussed here focus on a circumscribed study or group of studies, and plans for further development cannot be formulated without the results of these studies. Therefore, an exploratory IND application should articulate the rationale for selecting a compound (or compounds) and for studying them in a single trial or related trials, as this represents all that is known about the overall development plan at this stage. This section should also make it clear that the IND is intended to be withdrawn[8] after completion of the outlined study or studies.

2. Types of studies

Potentially useful study designs include both single- and multiple-dose studies. In single-dose studies, a sub-pharmacologic[9] or pharmacologic dose is administered to a limited number of subjects (healthy volunteers or patients). For example, microdose studies usually involve the single administration of a small dose with the goal of collecting pharmacokinetic information or performing imaging studies, or both.

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[8] The withdrawn, or inactive, IND can be referenced in any subsequent traditional IND.

[9] A radiolabeled candidate compound can be administered at doses that are known to have no pharmacologic effect in humans without an IND application in basic research studies when the compound has previously been studied in humans and the results published in the literature. These basic research investigations are conducted under the oversight of an institutional review board (IRB) and a radioactive drug research committee (21 CFR 361.1).

Repeat dose clinical studies can be designed with pharmacologic or pharmacodynamic endpoints. In exploratory IND studies, the duration of dosing should be limited (e.g., 7 days). For escalating dose studies done under an exploratory IND, dosing should be designed to investigate a pharmacodynamic endpoint, not to determine the limits of tolerability.

B. Chemistry, Manufacturing, and Controls Information

The regulations at 21 CFR 312.23(a)(7)(i) emphasize the graded nature of chemistry, manufacturing, and controls (CMC) information needed as development under an IND application progresses. Although in each phase of a clinical investigational program sufficient information should be submitted to ensure the proper identification, strength, quality, purity, and potency of the investigational candidate, the amount of information that will provide that assurance will vary with the phase of the investigation, the proposed duration of the investigation, the dosage form, and the amount of information already available. For the purpose of an exploratory IND application, the CMC information indicated below can be provided in a summary report to enable the Agency to make the necessary safety assessment.

The sponsor must state in the beginning of the exploratory IND application whether it believes the chemistry or manufacturing of the candidate product presents any potential for human risk (e.g., specific findings in preclinical studies associated with known risks of related compounds) (§ 312.23). If so, these potential risks should be discussed, and the steps proposed to monitor for such risks should be described.

The Agency is in the process of developing guidance explaining the stepwise approach to meeting current good manufacturing practice (CGMP) regulations. Once finalized, that guidance will be useful to persons seeking to manufacture, or prepare, products intended for use in an exploratory IND study.

1. General information for the candidate product

Except as noted below, the extent and type of chemistry and manufacturing information to be submitted in an exploratory IND application is similar to that described in current guidance for use of investigational products.[10] Information on each candidate product (i.e., the active ingredient) can be submitted in a summary report containing the following items.

- Description of the candidate product, including physical, chemical, and/or biological characteristics, as well as its source (e.g., synthetic, animal source, plant extract, or biotechnology-derived) and therapeutic class (e.g., radiopharmaceutic, immunosuppressant, agonist, antagonist) (see sections below for exceptions).
- Description of the dosage form and information related to the dosage form

[10] See guidance for industry *Content and Format of Investigational New Drug Applications for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-Derived Products.*

- Description of the formulation or routes of administration intended to be used in the human trial. For oral administration, sponsors can consider using suspensions or solutions in addition to the more usual tablets, powders, and capsules. For products intended for ophthalmic, inhalational (aqueous base), or parenteral administration, sterility and apyrogenicity must be ensured. For biological candidate products, freedom from contaminants associated with their manufacture, such as viruses, mycoplasma, and foreign DNA, also should be ensured. All excipients should be generally recognized as safe[11] or part of a formulation that is approved or licensed in the United States for the same route of administration and amount,[12] or adequately qualified through appropriate animal studies.
- The grade and quality (e.g., USP, NF, ACS) of excipients used in the manufacture of the investigational candidate product, including both those components intended to appear in the product and those that may not appear, but that are used in the manufacturing process
- Name and address of the manufacturer(s) (if different from the sponsor)
- The method of preparation of the candidate product lots used in preclinical studies and intended for the proposed human study, including a brief description of the method of manufacture and the packaging procedure, as appropriate, with a description of the container and closure system. For the active substance, include a list of the starting materials, reagents, solvents, catalysts used, and purification steps employed to prepare the candidate product. For sterile products, describe the sterilization process and controls for ensuring sterility. For biological/biotechnology-derived products, also identify the source material (e.g., Master Cell Bank), describe the expression system (e.g., fermentation methods) and harvest methods, as well as methods for removal/inactivation of potential viral contaminants. We recommend the use of a detailed flow diagram that includes all materials used as the usual, most effective, presentation of this information.
- Quantitative composition of the product
- A brief description of adequate test methods used to ensure the identity, strength, quality, purity, and potency accompanied by the test results, or a certificate of analysis, of the candidate product lots used in toxicological studies and intended for the proposed human study. For biotechnology products produced in mammalian cells or animals, this will include tests and studies to ensure the removal and/or inactivation of potential viral contaminants.
- Information that demonstrates the stability of the product during toxicology studies and an explanation of how stability will be evaluated during the clinical studies

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[11] Excipients considered to be generally recognized as safe (GRAS) are included in a list that is maintained on the Internet at http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm. See also 21 CFR 330.1, which explains the GRAS concept.

[12] Novel excipients should be appropriately qualified for their intended use. FDA has issued guidance on *Nonclinical Studies for Development of Pharmaceutical Excipients*.

- For ophthalmic, inhalational (aqueous base), or parenteral dosage forms, results from sterility and pyrogenicity tests

2. *Analytical characterization of candidate product*

There are two scenarios under which CMC information can be provided to an IND application. In the first scenario, the ***same batch*** of candidate product is used in both the toxicology studies and clinical trials. This material will be qualified for human use based on the CMC information (see III.B.1, above) and results of the toxicology studies described elsewhere in this guidance. Although we recommend establishing the impurity profile to the extent possible for future reference and/or comparison, not all impurities of the candidate product may need characterization at this stage of product development. If an issue arises during the toxicology qualification of the product, the appropriate parameters can be studied further, on an as-needed basis. Impurities (e.g., chemical and microbiological) should be characterized in accordance with recommendations in Agency guidance,[13] if, and when, the sponsor files a traditional IND for further clinical investigation.

In the second scenario, the batch of candidate drug product to be used in the clinical studies may not be the same as that used in the nonclinical toxicology studies. In such a case, the sponsor should demonstrate by analytical testing that the batch to be used is ***representative*** of batches used in the nonclinical toxicology studies. To achieve this, relevant analytical quality test results should be sufficient to enable comparison of different batches of the product. Tests to accomplish this include:

- Identity
- Structure (e.g., optical rotation (for chiral compounds), reducing/non-reducing electrophoresis (for proteins))
- Assay for purity
- Impurity profile (e.g., product- and process-related impurities, residual solvents, heavy metals)
- Assay for potency (biologic)
- Physical characteristics (as appropriate)
- Microbiological characteristics (as appropriate)

C. Safety Program Designs — Examples

Pharmacology and toxicology information is derived from preclinical safety testing performed in animals and in vitro. Preclinical studies for small molecules are described in ICH M3 while those for biologics follow guidance described in ICH S6. Some of the toxicology tests described in this guidance may not be appropriate for biologics. The toxicology evaluation recommended for an exploratory IND application is more limited than for a traditional IND application.[14] The

[13] See footnote 10 and guidance for industry, *INDs for Phase 2 and Phase 3 Studies, Chemistry, Manufacturing, and Controls Information.*

[14] International Conference on Harmonisation (ICH) guidance for industry *M3 Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals* describes what is expected for a traditional IND.

basis for the reduced preclinical package is the reduced scope of an exploratory IND clinical study. Although exploratory IND studies in some cases are expected to induce pharmacologic effects, they are not designed to establish maximally tolerated doses. Furthermore, the duration of drug exposure in exploratory IND studies is limited. The level of preclinical testing performed to ensure safety will depend on the scope and intended goals of the clinical trials.

There are a number of study objectives for which the preclinical safety programs may be tailored to the study design. Examples include: confirming that an expected mechanism of action can be observed in humans; measuring binding affinity or localization of drug; assessing PK and metabolism; comparing the effect on a potential therapeutic target with other therapies. Three examples are discussed in detail in the following paragraphs.

1. Clinical studies of pharmacokinetics or imaging

Microdose studies are designed to evaluate pharmacokinetics or imaging of specific targets and are designed not to induce pharmacologic effects. Because of this, the risk to human subjects is very limited, and information adequate to support the initiation of such limited human studies can be derived from limited nonclinical safety studies. A *microdose* is defined as less than $1/100^{th}$ of the dose of a test substance calculated (based on animal data) to yield a pharmacologic effect of the test substance with a maximum dose of ≤100 micrograms (for imaging agents, the latter criterion applies).[15] Due to differences in molecular weights as compared to synthetic drugs, the maximum dose for protein products is ≤30 nanomoles.

FDA currently accepts the use of extended single-dose toxicity studies in animals to support single-dose studies in humans. For microdose studies, a single mammalian species (both sexes) can be used if justified by in vitro metabolism data and by comparative data on in vitro pharmacodynamic effects. The route of exposure in animals should be by the intended clinical route. In these studies, animals should be observed for 14 days post-dosing with an interim necropsy, typically on day 2, and endpoints evaluated should include body weights, clinical signs, clinical chemistries, hematology, and histopathology (high dose and control only if no pathology is seen at the high dose). The study should be designed to establish a dose inducing a minimal toxic effect, or alternatively, establishing a margin of safety. To establish a margin of safety, the sponsor should demonstrate that a large multiple (e.g., 100X) of the proposed human dose does not induce adverse effects in the experimental animals. Scaling from animals to humans based on body surface area can be used to select the dose for use in the clinical trial. Scaling based on pharmacokinetic/pharmacodynamic modeling would also be appropriate if such data are available.

Because microdose studies involve only single exposures to microgram quantities of test materials and because such exposures are comparable to routine environmental exposures, routine genetic toxicology testing is not needed. For similar reasons, safety pharmacology studies are also not recommended.

[15] See European Medicines Agency (EMEA). Evaluation of Medicines for Human Use. "Position Paper on Non-Clinical Safety Studies to Support Clinical Trials with a Single Microdose." CPMP/SWP/2599/02Rev 1, 23 June 2004.

2. *Clinical trials to study pharmacologically relevant doses*

A second example involves clinical trials designed to study pharmacologic effects of candidate products. More extensive preclinical safety data would be needed to support the safety of such studies. However, since the goal would not include defining a maximally tolerated dose, the evaluation can still be less extensive than typically needed to support a traditional IND application. See the flow chart in the Attachment to this document.

Repeat dose clinical trials lasting up to 7 days can be supported by a 2-week repeat dose toxicology study in a sensitive species accompanied by toxicokinetic evaluations. The goal of such a study would be to select safe starting and maximum doses for the clinical trial. The rat is the usual species chosen for this purpose, but other species might be selected. In addition to studies in a rodent species, additional studies in nonrodents, most often dogs, can be used to confirm that the rodent is an appropriately sensitive species. If it is known that a particular species is most appropriate for a class of compounds, studies can be limited to that species. This confirmation can be approached in a number of ways. A lack of gender difference in the rodent study can serve as a basis for testing only a single sex in the second species if only a single sex will be studied in the clinical trial.

The numbers of animals used in the confirmatory study can be fewer than normally used to attain statistically meaningful comparisons, but of sufficient number to rule out any toxicologically significant difference in sensitivity compared with rodent (e.g. four non-rodents per treatment group). The confirmatory study could be a dedicated study involving repeat administrations of a single dose level approximating the rat NOAEL[16] calculated on the basis of body surface area. Alternatively, the test in the second species could be incorporated as part of an exploratory, dose escalating study culminating in repeated doses equivalent to the rat NOAEL. The number of repeat administrations at the rat NOAEL should, at a minimum, be equal to the number of administrations, given with the same schedule, intended clinically. The route of administration should be the same as the expected clinical route, and toxicokinetic measurements should be used to assess exposure. The same endpoints assessed in the rodent study should be evaluated in the second species. If the data from the confirmatory study suggest that the rodent is not the more sensitive species, a 2-week repeated dose toxicity study should be performed in the second species to select doses for human trials. This study should include measurements of body weight, clinical signs, clinical chemistries, hematology, and histopathology.

In contrast to microdose studies, for clinical trials designed to evaluate higher or repeated doses, each candidate product to be tested should be evaluated for safety pharmacology.[17] Evaluation of the central nervous and respiratory systems can be performed as part the rodent toxicology studies while safety pharmacology for the cardiovascular system can be assessed in the nonrodent species, generally the dog, and can be conducted as part of the confirmatory or dose-escalation study.

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[16] No-observed-adverse-effect level (NOAEL).

[17] For details see the guidance for industry *S7A Safety Pharmacology Studies for Human Pharmaceuticals*.

In general, each product in this type of exploratory IND should be tested for potential genotoxicity unless such testing is not appropriate for the population (e.g. terminally ill patients) or product to be studied. The genetic toxicology tests should include a bacterial mutation assay using all five tester strains with and without metabolic activation[18] as well as a test for chromosomal damage either in vitro (cytogenetics assay or mouse lymphoma thymidine kinase gene mutation assay) or in vivo. The in vivo test can be a micronucleus assay performed in conjunction with the repeated dose toxicity study in the rodent species. The high dose in this case should be a maximally tolerated or limit dose.

The results from the preclinical program can be used to select starting and maximum doses for the clinical trials. The starting dose is anticipated to be no greater than 1/50 of the NOAEL from the 2-week toxicology study in the sensitive species on a mg/m^2 basis. The maximum clinical dose would be the lowest of the following:

- ¼ of the 2-week rodent NOAEL on a mg/m^2 basis
- Up to ½ of the AUC at the NOAEL in the 2-week rodent study, or the AUC in the dog at the rat NOAEL, whichever is lower
- The dose that produces a pharmacologic and/or pharmacodynamic response or at which target modulation is observed in the clinical trial
- Observation of an adverse clinical response

Escalation from the proposed maximal clinical dose should only be performed after consultation with and concurrence of the FDA.

It is recognized that the studies described above are most appropriate for chemical drugs. Other animal models (e.g. nonhuman primates) may be more appropriate for biologics, and some tests may be inappropriate (e.g. genetic toxicology testing) for proteins.

3. *Clinical studies of MOAs related to efficacy*

A third example involves clinical studies intended to evaluate mechanisms of action (MOAs). To support this approach, the FDA will accept alternative, or modified, pharmacologic and toxicological studies to select clinical starting doses and dose escalation schemes. For example, short-term, modified toxicity or safety studies in two animal species based on a dosing strategy to achieve a clinical pharmacodynamic endpoint can in some instances serve as the basis for selecting the safe clinical starting dose for a new candidate drug. These animal studies would incorporate endpoints that are mechanistically based on the pharmacology of the new chemical entity and thought to be important to clinical effectiveness. For example, if the degree of saturation of a receptor or the inhibition of an enzyme were considered possibly related to effectiveness, this parameter would be characterized and determined in the animal study and then used as an endpoint in a subsequent clinical investigation. The dose and dosing regimen determined in the animal study would be extrapolated for use in the clinical investigation. In some cases, a single species could be used if it were established as the most relevant species based on scientific evidence using the specific candidate intended for the clinical investigation.

[18] *For* details see guidance for industry *S2A: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals* and *S2B: Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals.*

Although the production of frank toxicity is not the primary intended goal of the nonclinical study, relevant informative endpoints (e.g., hematology and histopathology) selected as important for clinical safety evaluation should be investigated. For example, an antibody that binds with a high degree of selectivity to a tumor-associated antigen could be studied in accordance with this third category. The mechanism of action of antibody-based products is generally associated with their binding properties and the effect on functions associated with immunoglobulins. Pharmacology and toxicology studies provide information about the selection of doses used in clinical studies through evidence of both a safe upper and potentially efficacious lower limit of exposure. These doses might be consistent with target plasma levels of the drug based on animal models of disease. The upper safe levels could be established in animal studies that show a lack of toxicity at these levels.

D. GLP Compliance

It is expected that all preclinical safety studies supporting the safety of an exploratory IND application will be performed in a manner consistent with good laboratory practices (GLP) (21 CFR Part 58). The GLP provisions apply to a broad variety of studies, test articles, and test systems. Sponsors are encouraged to discuss any need for an exemption from GLP provisions with the FDA prior to conducting safety related studies, for example, during a pre-IND meeting. Sponsors must justify any nonconformance with GLP provisions (21 CFR 312.23(a)(8)(iii)).

IV. CONCLUSION

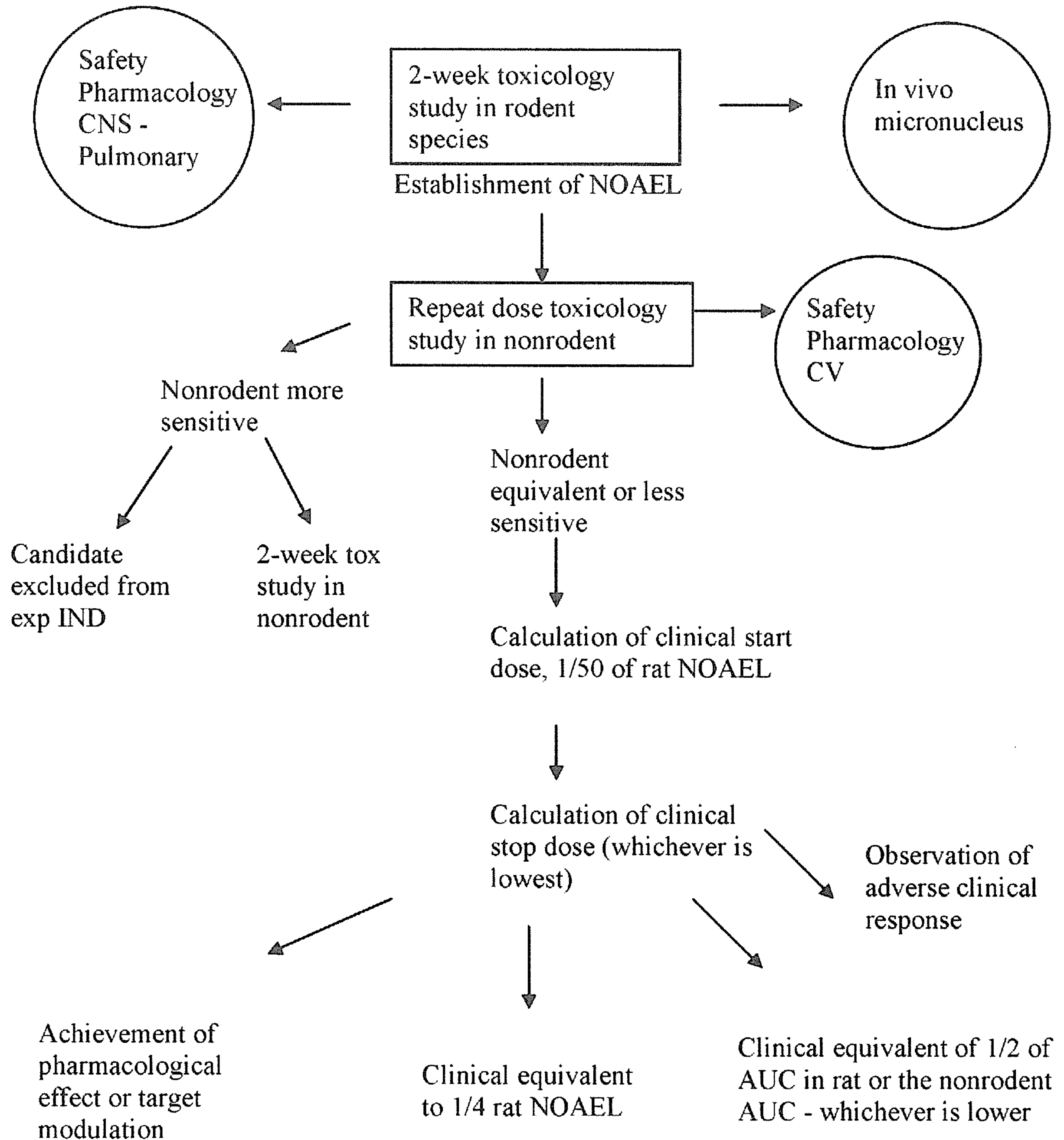
Existing regulations allow a great deal of flexibility in the amount of data that needs to be submitted with any IND application, depending on the goals of an investigation, the specific human testing being proposed, and the expected risks. Sponsors have not taken full advantage of that flexibility, and limited, early phase 1 studies, such as those described in this guidance, are often supported by a more extensive preclinical database than is needed for those studies alone.

The common theme throughout this guidance is that, depending on the study, the preclinical testing programs for exploratory IND studies can be less extensive than for traditional IND studies. This is because for the approaches discussed in this guidance, which involve administering sub-pharmacologic doses of a candidate product or products, the potential risks to human subjects are less than for a traditional phase 1 study.

The Agency is undertaking a number of efforts to reduce the time spent in early drug development on products that are unlikely to succeed. This guidance describes some exploratory approaches that are consistent with regulatory requirements, but that will enable sponsors to move ahead more efficiently with the development of promising candidate products while maintaining needed human subject protections.

ATTACHMENT

A Preclinical Toxicology Testing Strategy for Exploratory INDs Designed To Administer Pharmacologically Active Doses



Development of Novel Advanced Cell and Gene Therapy and GMP-Controlled Cell Processing

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Abstract: High scientific and ethical practices and reliability in compliance with the ICH-GCP (International conference on harmonization-good clinical practices) are properly required for clinical trials/research involving human subjects. Cell therapy is a general term for treatment modalities conducted by transplantation of human cells, such as blood transfusion, hemopoietic stem cell transplantation, cell transfer immunotherapy, gene therapy, and regenerative therapy. A production system called cell processing including human cell preparation, cultivation, and gene transduction is essential for the development of therapies using cells, and the quality control thereof must be performed in compliance with the good manufacturing practice (GMP). At the moment, however, Japan is far behind in the formulation of rules for cell processing, which should be evolved without delay in order to promote the development of advanced therapy. In particular, it is urgent to construct an institutional GMP (iGMP) specialized for academic institutions and centers, where development of advanced therapy such as regenerative therapy and cell therapy is being undertaken.

Key words: Development of novel advanced therapy; Cell therapy; Regenerative therapy; Gene therapy; Cell processing

Introduction

In view of the life science research evolving with remarkable progresses since the beginning of the 21st century, it is eagerly awaited that the results of basic research would be fruitfully translated into effective therapeutics for patients with incurable diseases. The term "cell therapy" includes much of regenerative therapy, immunotherapy and gene therapy that progress based on novel theories or strategies. High scientific and ethical practices and reliability are required for the development of such edge-cutting advanced therapies.

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Currently, drugs used in clinical settings are manufactured in compliance with the current good manufacturing practice (GMP), the standards for production and quality control of drugs. In Europe and the United States, GMP-controlled cell processing is mandatory for the development of translational research using human cells *per se* to treatment.

Rationale of the Need for GMP-Compliant Cell Processing

Development of a new drug, based on basic research data, proceeds to preclinical studies and further to phase I trials. The drug used in these studies should be produced under GMP control. In the States, an investigational new drug (IND) authorized by the Food and Drug Administration (FDA) is used in clinical trials, and a similar system is being implemented for drugs in Japan as well. Deduced from these principles, it is readily understandable that production of human cells for clinical use must also be in compliance with the GMP when those cells are considered to represent "cell pharmaceuticals".

The prototype of cell therapy is blood transfusion, and blood products are prepared in accordance with the GMP at blood centers or blood banks. It is essential that individual products be checked for potentially transmissible infectious agents since human cells, which unlike drugs do not constitute batches, are used. Most translational researches with the use of human cells are conducted at academic institutions and centers for novel advanced therapy. No one would allow the transplantation of cells prepared at a conventional laboratory without any defined standards or records.

The System in the United States and Current Status in Japan

In January 2001, the US FDA proposed the current good tissue practice (cGTP), which specifies those matters required for the production of human cells for therapeutic use, particularly to prevent transmission of infections, and finalized in November, 2004.[1] Further, the sterile drug products produced by aseptic processing was also finalized by the Center for Drug Evaluation and Research (CDER) in September 2004.[2]

It states, "Poor cGMP conditions at a manufacturing facility can ultimately pose a life-threatening health risk to a patient" in the introduction section, and provides concrete descriptions of an aseptic processing facility design layout, aseptic processing techniques and management. The CBER focuses primarily on the aseptic processing for drugs, but also gives consideration to applicability of the standards to cell processing.

In Japan, it is stipulated in the Amended Pharmaceutical Affairs Law enforced as of July 30, 2003, that "regarding 'Biological Products' which require advanced production process control, the premises and the procedures of production and quality control at the manufacturing facility (annotation by the author: the so-called GMP) shall comply with the manufacturing standards for ordinary drugs and medical devices and, in addition, shall comply with the supplementary standards laid down by the Ministry of Health, Labour and Welfare". A subordinative law will be issued in April, 2005.

FDA's Guiding Principles for Development of Advanced Cell Therapy

When intending to carry out development of cell therapy in the States, researchers or clinical investigators should prepare a clinical trial protocol and submit documents required for GMP-controlled cell processing to the FDA. The FDA evaluates the cells for therapy as an IND to be used in translational research, inspects the manufacturing facility, and guides the applicant in preparing GMP-related documents.

The FDA officer recognizes the disparity between the GMP for conventional drugs and

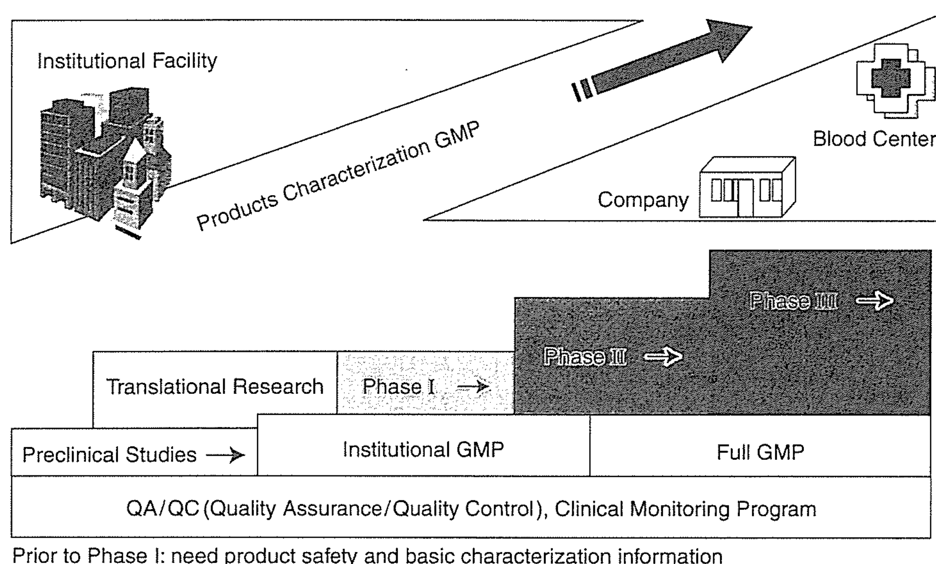

Fig. 1 Stepwise approach: regulatory requirements increase with product development
Institutional GMP should be established to advance translational research in academia

that required for cell processing, and is ready to lend his/her cooperation in establishing an institutional GMP necessary for translational research, with actual situations in academia taken into account, based on full GMP required for pharmaceutical manufacturers to follow in the production of commercial products (Fig. 1). Thus, in the States, a national strategic stance is being taken to actively aid academic institutions in the development of a novel therapy, concerning that the motivation for developmental research will be diminished because of the lack of clearly established standards.

Points at Issue in Japan

There is an opinion that "construction of GMP-controlled cell processing may well be left to the hands of enterprises or companies". It is anticipated, indeed, that manufacturers can make inroads into the area such as cultured skin, where products are already in clinical application. However, what are developed in academia or centers for advanced therapy are for the most part those products manufactured through translational research based on results of basic research, and are yet to be determined as to whether they can be established as novel therapeutics.

It is difficult for a pharmaceutical company to make a positive entry into the development of GMP-controlled cell processing without running a risk at such a stage of translational research. Therefore, researchers, clinical investigators, pharmacists, medical technologists (biologists), engineers, GMP consultants, and researchers of corporate advanced medicinal development divisions should closely collaborate in constructing the standards based on global rules specialized for academia and centers for advanced therapy.[3]

Summary and Conclusion

It has become recognized at length in Japan that the cell processing control bodies must be ready for the development of cell therapy and regenerative therapy. However, some institutions are still carrying out parallel incubation of cells from several different individuals within the same incubator with a clean bench settled in a conventional laboratory, or are performing

co-cultivation with murine cells or administration of cells grown in cultures with fetal bovine serum simply because such has been approved by a local ethical committee. No one would allow, even though indirectly, a transplantation of or being injected with an article which one even hesitates to eat.

There is no law to regulate such a situation at present in Japan. GMP-controlled cell processing is thus definitely needed all the more for culturing human cells and performing gene transfection in the advanced medical treatment where safety and efficacy have not been fully verified, especially in cell therapy and regenerative therapy. This constitutes basic rules that all individuals engaged in the development of advanced medical treatment must observe. Stringent regulation is required all the more in such experimental exploratory treatment of which safety and therapeutic effects are yet to be established. Research and development starting in a slovenly manner will yield irreparable results. It should be done in strict accordance with regulations until "the cell processing is thought to be infallibly safe up to the proven level", so that due deregulation then may gradually follow.

Establishment of institutional GMP for academia and centers for advanced therapy is mandatory for the development of advanced therapy in terms of cell therapy, and a keen insight from those concerned are greatly expected.

Acknowledgment

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高度先進医療部門

笠井泰成[1)]/前川　平[2)]

〔SUMMARY〕 高度先進医療は数多くの基礎研究と技術開発を礎として，その成果の上に成り立っている．なかでも，最新の知識や技術を集約した細胞治療や再生治療は，専門化された教育訓練を受けた多くの医療スタッフにより構成され，実施されるチーム医療でもある．臨床検査技師にとっても専門的な知識や技術だけでなく，生命倫理に対する確固たる見識が必要とされ，これらの先進医療開発分野も新たな活躍の場として期待できる．〔臨床検査　49：872-873，2005〕

〔KEYWORDS〕 高度先進医療，チーム医療，専門化教育

はじめに

臨床検査技師の教育制度も以前は短期大学や専門学校が主体であったが，短期大学の多くが4年制の大学へと改組されている．さらに大学院へ進学も可能となり，より専門的な知識や技術の習得が可能となってきた．多くの知識や技術を学んだ学生が，大学卒業後には医療の現場だけでなく，医療に関連した様々な分野へと拡がり，活躍できるような状況へと変わりつつある．臨床検査も一般的な検査技術ばかりでなく，特殊な技術を応用したものが開発され実用化が進められている．このような状況の中で臨床検査技師は，単に臨床側から依頼された検査の結果を返すだけでなく，臨床の現場にも自ら出向き，検査結果の解析や検査の進め方についてアドバイスをするなど，ベッドサイドで医療スタッフの一員としての積極的な活動が望まれる．また，新しい検査方法や検査試薬の開発だけでなく，高度先進医療開発への参画など，多方面での活躍も期待できる．

高度先進医療は，数多くの基礎研究と技術開発を礎として，その成果の上に成り立っている．最新の知識や技術が集結された高度先進医療は，高度に専門化された教育訓練を受けた多くの医療スタッフにより構成され，実施されるチーム医療でもあり，臨床検査技師にとっても新たな活躍の場として期待できる分野である．

細胞治療

高度先進医療といっても様々な分野があるが，その1つに細胞治療が挙げられる．細胞治療には，疾病や傷害などによりヒトの体の一部が物理的に失われたり，機能の一部が失われた場合に，自然には再生できない臓器，組織，細胞などを間葉系幹細胞などから新しく作り出し，損失した機能を回復させようとする再生医療や，特定の遺伝子を組み込んだ細胞を用いて疾病の治療を行おうとする遺伝子治療などが含まれる．十分な治療方法がいまだ確立されていない難治疾患に苦しむ人々は，このような新しい再生治療や細胞治療の開発を今や遅しと待ち望んでいる．米国での調査では，細胞治療の適応対象になると予測される患者数が米国国内にも数百万人以上が存在すると報告されている(表1)[1)].

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0485-1420/05/¥250/論文/JCLS

表1　ヒト多能性幹細胞の研究により救援できる可能性のある疾患に罹患している患者数(米国国内での集計)

疾患名	患者数(単位：百万人)
心血管症	58
自己免疫症	30
糖尿病	16
骨粗鬆症	10
癌	8.2
アルツハイマー病	4
パーキンソン病	1.5
重症熱傷	0.3
脊髄損傷	0.25
先天異常	0.15
合計	128.4

〔文献1)より引用〕

このような再生治療や細胞治療を行うためには，治療用のヒト細胞を作製する必要がある．すなわち，ヒト由来の組織や細胞を分離・調整・培養などの操作を行う細胞プロセッシングがなければこれらの開発はできない．細胞治療に用いられる組織や細胞は，疾患の治療を目的として再び人の体内に戻される．そのため細胞プロセッシングの工程には，医薬品の製造と同じように高い安全性と十分な品質管理が求められる．医薬品などの製造販売に関しては，GMP(Good Manufacturing Practice)に準拠した管理基準が法的に定められているが，細胞プロッセシングについても同様に厳格な品質管理や製造管理が必要となることは容易に理解できる[2]．

高度先進医療とチーム医療

細胞治療などの臨床試験研究の統括責任者には，一般的に医師などが担当することになるが，高度先進医療の開発やその実施には医師だけでなく，その内容に応じて様々な職種の医療スタッフが必要となってくる．

細胞プロセッシングにおいて，高い品質と安全性が保証された操作を行うためには根拠に基づいた知識と熟練した技術を身につけたスタッフが不可欠である．例えば，無菌操作による細胞分離や細胞培養などである．さらに，関連した最新の情報をつねに収集して必要な情報を取り入れ，品質の向上を目指さなければならない．また，細胞プロセッシングを受けた組織や細胞の品質管理や機能検査を行うために様々な検査が必要となるが，コストパフォーマンスを考慮しながら適切な検査方法を選択し，その検査結果を正確に評価することも必要となる．このようなポジションには，高度に専門化された教育を受けた臨床検査技師が適しているといえる．もちろん，このような教育訓練は大学での教育のみですべてを習得することは困難であり，卒後教育の一環として設置される専門教育のトレーニングコースも受講していかなければならない．

また，細胞プロッセッシングを行うには，専用のクリーンルームを設置したり，特殊な装置が必要となる．これらの清浄度や無菌状態を維持するには日常的な点検や整備などの管理が重要な業務となってくるが，医師や看護師がこれらの管理業務を行うことは適切とはいえない．これらの設備や装置の構造および仕組みなどを十分に理解し，常に的確な操作ができるようにしておくことも，臨床検査技師に課せられた責務である．

専門職として

臨床検査技師が活動領域を拡げていくには，臨床検査技師が専門職として認められることが必要条件であると考える．そのためには，(1)臨床検査技師が個人の責任を伴う知的活動を行い，共に業務に従事する者との間にグループ意識を築けるような活動，職務を行うこと，(2)臨床検査技師が行う業務は，学問的，理論的であり，その目的において実務的でもあること，そして(3)その技術は高度に専門化された教育訓練を通して，はじめて取得できるものであることなどが必要である．これからの臨床検査技師はTechnicianであると同時に，Technologistあるいは Biologistでもあることが望まれる．加えて，先端医療開発にかかわる検査技師にも生命倫理に対する確固たる見識が要求されるようになると考えている．

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Prospective Characterization of Neural Stem Cells by Flow Cytometry Analysis Using a Combination of Surface Markers

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Neural stem cells (NSCs) with self-renewal and multilineage differentiation properties can potentially repair degenerating or damaged neural tissue. Here, we have enriched NSCs from neurospheres, which make up a heterogeneous population, by fluorescence-activated cell sorting (FACS) with antibodies against syndecan-1, Notch-1, and integrin-β1, which were chosen as candidates for hematopoietic cell—or somatic stem cell—markers. Antigen-positive cells readily initiated neurosphere formation, but cells lacking these markers did so less readily. Doubly positive cells expressing both syndecan-1 and Notch-1 underwent neurosphere formation more efficiently than did singly positive cells. The progeny of sorted cells could differentiate into neurons and glial cells both in vitro and in vivo. These antibodies were also useful for isolating cells from the murine embryonic day 14.5 brain that efficiently formed neurospheres. In contrast, there was no distinct difference in neurosphere formation efficiency between Hoechst 33342-stained side population cells and main population cells, although the former are known to have a stem cell phenotype in various tissues. These results indicate the usefulness of syndecan-1, Notch-1, and integrin-β1 as NSC markers. © 2005 Wiley-Liss, Inc.

Key words: neurosphere formation ratio (NFR); enrichment value (EV); syndecan-1; Notch-1integrin-β1

In the vertebrate central nervous system, the periventricular region, particularly the subventricular zone (SVZ), persists in neonatal and adult mammals as a mitotically active layer (Luskin, 1993; Levison and Goldman, 1993). It is now apparent that the SVZ of the lateral ventricles and the hippocampal granule cell layer of adult rodents are late germinal matrices whose molecular and cellular microenvironment is able to sustain neurogenesis via the proliferation of undifferentiated multipotent neural precursors (Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Kuhn et al., 1996; Doetsch et al., 1997).

However, the molecules and mechanisms controlling neural stem cell (NSC) or progenitor cell proliferation are only partially understood. Epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) influence the extent and rate of proliferation of NSC and progenitor cell populations in vitro and in vivo (Reynolds and Weiss, 1992; Richards et al., 1992; Vescovi et al., 1993; Gage et al., 1995; Palmer et al., 1995; Kuhn et al., 1997) and are mitogens for mouse-derived neurospheres (Reynolds and Weiss, 1996; Gritti et al., 1996). The use of the neurosphere method has even allowed the selective expansion of NSCs from human neural precursor cells (Svendsen et al., 1998; Kanemura et al., 2002), and their clinical application has been discussed (Okano, 2002a,b).

Over the past few decades, hematopoietic stem cells (HSCs) and progenitor cells have been identified by using monoclonal antibodies directed against their surface markers, which allows rare populations of cells to be enriched while remaining viable (Uchida and Weissman, 1992; Morrison and Weissman, 1994; Osawa et al., 1996; Uchida et al., 1996; Akashi et al., 2000). In contrast to the detailed studies that have advanced our understanding of HSCs, the lack of effective methodologies for the prospective identification or purification of NSCs has slowed research into their biology, although NSCs have come to be defined experimentally as neurosphere-initiating cells (NS-ICs; Reynolds and Weiss, 1992). Although selective NSC markers, such as Musashi1 (Sakakibara et al., 1996; Sakakibara and Okano, 1997; Kaneko et al., 2000), nestin (Hockfield and McKay, 1985; Lendahl et al., 1990), and Sox1 (Pevny et al., 1998), have been developed, antibodies against these intracellular molecules cannot be used for directly iso-

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lating live cells. The first example of immunoselection using a surface antigen was reported by Johansson et al. (1999), who used an antibody to Notch1 to enrich NSCs from the adult mouse brain. Subsequently, Uchida et al. (2000) succeeded in isolating a population enriched for human fetal NSCs by sorting $CD133^+$ $CD34^-$ $CD45^-$ cells. Alternatively, Rietze et al. (2001) reported the isolation of one type of adult mouse NSC from the periventricular area by selecting cell size and the expression of peanut agglutinin-binding activity, a marker for mouse HSCs (Salner et al., 1982). Some of these cells expressed the marker Lewis-1 (Capela and Temple, 2002) but only low levels of CD24. Here, we have systematically evaluated the expression of several cell surface markers specific to HSCs or progenitor cells, or other somatic tissue stem cells, in populations of dissociated neurosphere cells and fetal brain tissue cells. This analysis subsequently allowed us to measure the frequency of NS-ICs by limiting dilution culture after fluorescence-activated cell sorting (FACS).

MATERIALS AND METHODS

Primary Culture

C57BL/6 embryonic mice were sacrificed at day 14.5 (E14.5) by cervical dislocation. The striatum, or SVC, of the embryonic telencephalon was dissected and mechanically dissociated by gentle pipetting into single cells, which were cultured in DMEM/F12 1:1 serum-free medium (SFM; Sigma, St. Louis, MO) containing 15 mM HEPES (Nacalai tesque), 0.6% glucose, 25 μg/ml insulin (Roche, Indianapolis, IN), 100 μg/ml transferrin (Roche), 60 nM progesterone, 60 μM putrescine, 30 nM sodium selenite, and 20 ng/ml EGF (Sigma), at 37°C in a 5% CO_2 atmosphere in a humidified incubator for 5–7 days. The cell density was $2.0–4.0 \times 10^5$ cells/ml. EGF equivalent to a final concentration of 20 ng/ml was added every other day.

For transplantation we used embryonic green fluorescent protein (GFP) mice (a gift of Dr. Okabe, Osaka University; Okabe et al., 1997). In these mice, GFP is expressed as a reporter gene in various tissues, including the neural system, and during all developmental stages. The striatum cells dissected from these mice were cultured in the same manner as described above.

Determination of the Frequency of NS-ICs Among Primary Neurosphere Cells

Primary neurospheres were harvested at 5–7 days in vitro (DIV) by centrifugation at 1,000 rpm for 3 min and mechanically dissociated into single cells by gently pipetting in 1% bovine serum albumin (BSA)/phenol-red free SFM (Gibco, Grand Island, NY). The cells were then centrifuged at 1,500 rpm for 5 min, blocked with anti-FcγIII/II receptor (CD16/CD32) antibody (PharMingen, San Diego, CA) and labeled with primary and secondary antibodies for 30 min each at 4°C. The selected primary antibodies were against syndecan-1 (CD138; rat monoclonal 281-2), Notch-1 (Santa Cruz Biotechnology, Santa Cruz, CA; rabbit polyclonal), integrin-β1 chain (CD29; hamster monoclonal Ha2/5), Thy1.2 (CD90.2; rat monoclonal 30-H12), Sca-1, c-kit, CD34, H2kb, CD45.2, VEcad, Flk-1, CD49d, TER 119/Ly76, AA4.1, Mac-1, CD19, B220, CD31, Ly9.1, and Gr-1. All antibodies were obtained from PharMingen, except where otherwise noted. The primary antibodies were evaluated for stable expression on neurosphere cells at least three times by using a FACS Caliber (Becton Dickinson, San Jose, CA). When the primary antibody was not labeled directly, an appropriate secondary labeled antibody was used depending on the primary antibody used. Primary or secondary antibodies used for detection were labeled with phycoerythrin or fluorescein isothiocyanate (FITC).

Three populations, including the positive, the negative, and the unlabeled live cell populations (control) for each antibody, were sorted. For the integrin-β1- or Thy-1-positive population, cells at 10–20% of the highest fluorescent intensity were sorted. Each population was sorted into wells of 96-well plates with a FACS VantageSE and Clonecyt Plus software (Becton Dickinson). Dead cells were excluded by propidium iodide (PI) staining (3 μg/ml). Each well was first treated with 100 μl SFM and 20 ng/ml EGF, and then 5, 10, or 20 sorted cells were added. These cells were cultured without further EGF addition for 21 DIV, to allow for limiting dilution analysis. Time-course analysis revealed that a maximal, plateau level of neurosphere formation ratio (NFR) was achieved at about 21 DIV.

To enrich further for NS-ICs, three combinations of antigens were tested, syndecan-1 and integrin-β1 (S/I), integrin-β1 and Notch-1 (I/N), and Notch-1 and syndecan-1 (N/S). Four populations; the doubly positive, the singly positive, and the doubly negative for each combination, as well as the total live cells (control), were sorted simultaneously. Wells of 96-well plates were seeded with 2, 5, or 10 sorted cells, which were cultured and analyzed as described above.

After 21 DIV, the number of wells (A) that contained at least one neurosphere and the total number of wells (B) seeded with sorted cells were determined. "Neurosphere" was defined as a floating sphere greater than 50 μm in diameter and could be distinguished from two-dimensional aggregates of cells. To assess the efficiency of neurosphere formation, the NFR, which was defined as $(A/B) \times 100$, was compared for all cases. The frequency of NS-ICs was determined by the limiting dilution method.

Determination of the Frequency of NS-ICs Among Primary Fetal Brain Tissue Cells

Primary tissue cells were sorted by using the combinations of the three antigens described above. Single cells dissociated from E14.5 mouse striatum were subjected to analysis together with another sample of dissociated primary neurosphere cells prepared after 5–7 DIV. Each sample was divided into four populations, of which three were labeled with the combined antibodies, S/N, I/N, and S/I, and the fourth was left unlabeled as a live cell control. The doubly positive fractions from the three labeled samples and the control were sorted, seeded at a density of 2, 5, or 10 cells/well, and analyzed in the same manner as described above.

Determination of the Enrichment Value (EV) Under Limiting Dilution Analysis

The neurosphere formation efficiency, represented as the NFR in this study, was determined by a linear regression method with limiting dilution. The number of NS-ICs in each

well is random and follows the Poisson distribution (Tropepe et al., 1999). In this distribution, $F_0 = e^{-u}$, where F_0 is the proportion of wells without NS-ICs (described as "% well without neurosphere" in the figures), e is ~2.718, and u is the mean number of NS-ICs in each well. If there is one NS-IC in each well (u = 1), $F_0 = 0.37$. With this equation, the frequency of wells without a neurosphere is 37% under conditions in which every well contains one NS-IC. In this setting, the neurosphere formation value (NFV) a is defined as "the mean number of total sorted cells per well" for the control population. The NFV b is similarly defined, but with respect to the specified cell population. The neurosphere formation efficiency based on a linear regression analysis is represented by the index "enrichment value (EV)," which is defined as a to b (NFV a: the number of cells per well containing one NS-IC on average for each control population, NFV b: that for each positive population). Efficient neurosphere formation is reflected by a high value of EV. Similarly, for combination assays, a to b′ (NFV a: the number of cells per well containing one NS-IC on average in each control population, NFV b′: that in each doubly positive population) was determined.

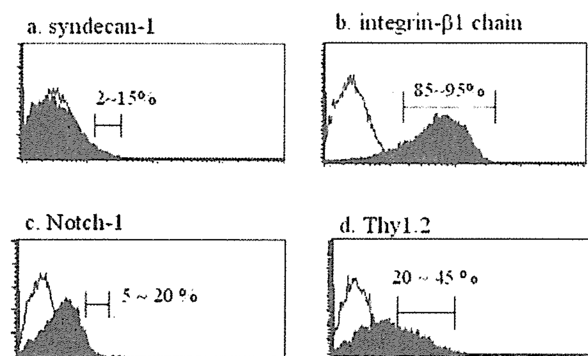

Fig. 1. **a–d:** The four antigens that were substantially expressed on neurosphere cells in screening. The neurosphere cells after culturing for 5–7 DIV were screened with the antibodies for the hematopoietic progenitor or somatic stem cells by FACS. The horizontal line shows the fluorescent intensity of each antigen, and the vertical line shows the amount of expression for each antigen. The numbers in the chart are the ratios of the positive-to-all live cells for each antigen. White area, expression of each isotype control; gray area, expression of the specified antigen.

Side Population Analysis

Single cells dissociated from primary neurosphere cells, which were cultured for 6 DIV in 20 ng/ml EGF-containing SFM, were resuspended in prewarmed 1% BSA/SFM at 1.0×10^6 cells/ml. Hoechst 33342 dye (Molecular Probes, eugene, OR) was added at 3 μg/ml with or without 50 μM verapamil. The cells were incubated in a humidified 5% CO_2 incubator at 37°C for 40 min in a prewarmed water bath, stirred by pipetting after 20 min during the incubation period, and washed with 1% BSA/SFM at 4°C after the incubation. After 30 min on ice, they were sorted using aFACS VantageSE after PI exclusion (3 μg/ml). Two populations, side population (SP) and main population (MP), were sorted, and the final dilutions were set at from 1 to 100 cells/well. Counting and analysis were performed as described above.

Statistical Analysis

The NFRs at every corresponding cell density between two properly set populations were analyzed by using the Wilcoxon *t*-test. A *P* value of <0.05 was accepted as the level of statistical significance (n = 3 or more at every cell density in all assays).

Neurosphere Differentiation and Immunocytochemical Staining

Primary or secondary neurospheres after 5–7 DIV were washed with EGF-free SFM and incubated in poly-L-ornithine (PLO)-coated four- or eight-well Permanox chamber slides (Nalge Nunc International, Naperville, IL), which contained 1% fetal calf serum in SFM at 37°C. At 4–6 DIV, the attached neurospheres were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min at 4°C and then treated with 0.2% Triton X-100/10% goat serum/PBS for 40 min at 25°C. They were then incubated with primary antibodies against glial fibrillary acidic protein (GFAP; Sigma; rabbit IgG) as an astrocyte marker, microtuble-associated protein-2 (MAP2; Chemicon, Temecula, CA; mouse monoclonal; AP20) as a neuronal marker or galactocerebroside (Galc; Chemicon; rabbit polyclonal) as an oligodendrocyte marker, or with appropriate pairs of these antibodies for double-immunocytochemical staining overnight at 4°C and washed with PBS three times for 20 min each. The cells were then incubated with an appropriate secondary antibody: rhodamine-conjugated goat anti-mouse IgG (Chemicon) or FITC-conjugated goat anti-rabbit IgG (Roche) for 1 hr at 25°C. The cells were also treated with Hoechst 33342 for 5 min where indicated in the text. After three washes, the chamber wall was removed, and the cells were coverslipped with Vectashield (Vector, Burlingame, CA) as the mounting medium. The cells were observed by fluorescence microscopy (Olympus IX70) and laser scanning microscopy (Olympus Fluoview).

About 50–100 primary neurospheres derived from the positive cells for each surface antigen were differentiated and doubly immunostained and were then examined for the three neural lineage markers using fluorescence microscopy as described above. The positive ratio (X/Y) was determined from the numbers of neurospheres (X) positive for each neural lineage-marker divided by the numbers of all examined neurospheres (Y) for each (%Galc, %GFAP, %MAP2).

Transplantation of Neurosphere Cells Derived From GFP Transgenic Mice

The striata of E12.5–14.5 embryos derived from GFP mouse parents were dissected and sorted as donor cells by FACS using anti-integrin-β1, syndecan-1, or Notch-1 antibodies after culturing for 5–7 DIV. The ratio of live cells to all sorted cells as determined by trypan blue exclusion was >80%.

Postnatal (day 0–3) C57BL/6 wild-type neonatal mice were used as recipients. Each neonate was deeply hypothermia-anesthetized on ice and injected with about 2.0×10^5 cells in a 2-μl volume into the lateral ventricle of the brain with a manual manipulator (Narishige IM-3 and GJ-1) equipped with a microsyringe and a 33-gauge needle (Ito or Hamilton, inner diameter 70–110 μm, outer diameter 200–210 μm, original length 10 mm,

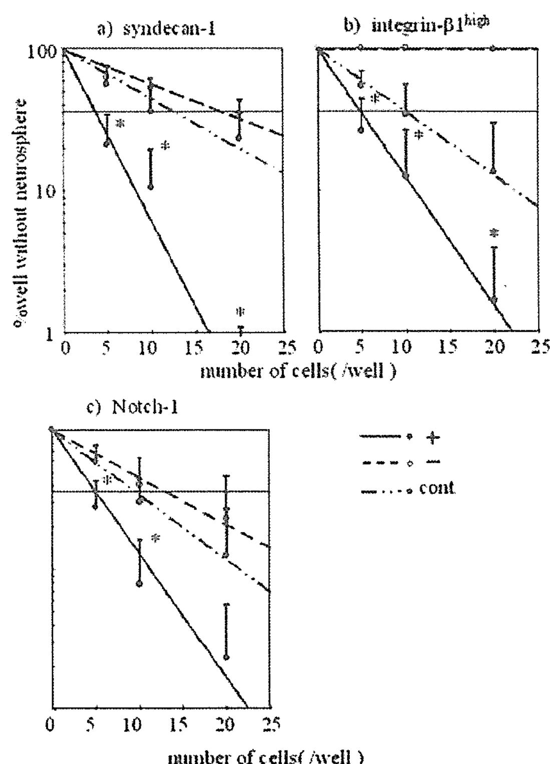

Fig. 2. **a–c:** Linear regression analysis with limiting dilution for the three antigens that showed the most efficiency. The neurosphere cells after 5–7 DIV were sorted by FACS using the three antibodies described above and analyzed after culturing for 21 DIV. The positive cells formed neurospheres significantly more efficiently than the controls (*$P < 0.05$). Solid line, positive cells; dashed and dotted line, control cells; dashed line, cells negative for each antigen.

TABLE I. Neurosphere Formation Values (NFVs) and Enrichment Values (EVs) for the Four Antigens*

	Syndecan-1	Integrin-β1	Notch-1	Thy1.2
NFV				
a	12.4	9.7	9.3	10.3
b	3.6	4.8	4.9	6.3
EV				
a/b	3.4	2.0	1.9	1.6

*The neurosphere cells after 5–7 DIV were sorted using these antigens and analyzed after culturing for 21 DIV. The NFV *a (the number of cells per well that contained one NSC on average for each control population), b (that for the singly positive population of each antigen)* and EV *a/b* revealed that syndecan-1$^+$ cells formed neurospheres the most efficiently among all tested antigen-positive cells.

style 3) through a skin and cranial bone tract opened with a 27-gauge disposable needle (Nipro).

The recipient mouse was sacrificed 2–10 weeks posttransplantation by anesthesia with diethyl ether and fixed by transcardiac perfusion with ice-cold 4% PFA after PBS. The whole brain was dissected and immersion-fixed with 4% PFA for 30 min at 4°C and sectioned into 1–2-mm slices with a razor, and the tissue slices were fixed by reimmersion for 2–3 hr at 4°C. GFP-positive samples were incubated for two successive nights in 15% and 20% sucrose solution, and then in 30% sucrose solution for 24 hr. They were embedded in O.T.C. compound and frozen as 10–12-μm sections with a cryostat (Leica CM 1850). Immunohistochemical analysis was performed in a manner similar to that described above for immunocytochemical analysis. Each section was stained with primary antibodies against GFP (Clontech, Palo Alto, CA) as a marker of transplanted cell, NeuN (Chemicon), glutamic acid decarboxylase 65 (GAD65; Sigma), or γ-aminobutyric acid (GABA; Sigma) as neuronal markers; GFAP (Sigma) or glutamine synthetase (Sigma) as astrocyte and glial cell progenitor markers; myelin basic protein (MBP; Chemicon), 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP; Chemicon), or NG2 chondroitin sulfate proteoglycan (Chemicon) as markers of oligodendrocytes and their precursors; or nestin (Chemicon) as a neural progenitor marker. The proper secondary antibodies conjugated with rhodamine/Cy3 or FITC were used.

RESULTS

Establishment of an NSC Assay Using a Limiting Dilution Method

There is consensus that NSCs or progenitors in SFM in vitro proliferate to form neurospheres that differentiate into the three neural lineages. In our study, NS-ICs were considered to be cells that have NSC potency, as defined above. Too evaluate the frequency of NS-ICs, we established a method to measure systematically the neurosphere formation frequency using FACS and clone sorting (Engstrom et al., 2002) with limiting dilution culture. The final diluted cell concentration was set from 1 to 100 cells/well in a 100-μl volume. Time-course analysis revealed that a maximal, plateau level of the neurosphere formation rate was achieved at about 21 DIV. A linear regression analysis with limiting dilution assays revealed that the neurosphere formation value (NFV) was 21.8; that is, every 21.8 live cells contained an average of one NSC in our system (data not shown). Secondary neurospheres at 21 DIV could differentiate into cells of the three neural lineages (data not shown).

Expression of Surface Antigens on Neurosphere Cells

Recent reports suggested that HSCs and NSCs share some common characteristics. That is, HSCs and NSCs express some common antigens and/or genes (Uchida et al., 2000; Terskikh et al., 2001; Rietze et al., 2001; Ramalho-Santos et al., 2002) or have the potential to transdifferentiate into each other in vivo (Bjornson et al., 1999). We screened the expression of surface antigens, such as syndecan-1, Notch-1, integrin-β1, Thy1.2, Sca-1, c-kit, CD34, H2kb, VEcad, Flk-1, CD49d, AA4.1, CD31, and Ly9.1, which have been identified as characteristic of HSCs or somatic stem cells. In addition, we also examined CD45.2, TER 119/Ly76, Mac1, CD19, B220, and Gr-1, which are known to be specific for hematopoietic