

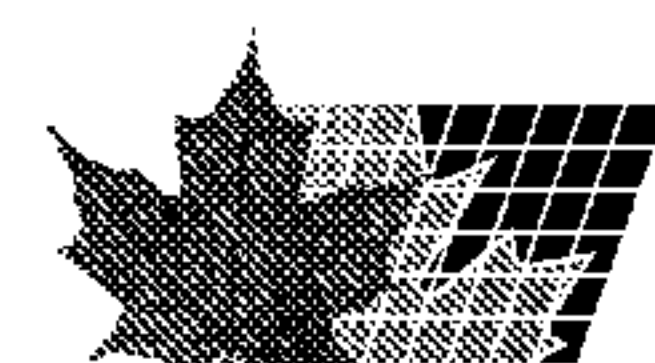


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(54) Title: METHODS OF DELIVERY OF EXOGENOUS PROTEINS TO THE CYTOSOL AND USES THEREOF

(57) **Abrégé/Abstract:**

The present invention is directed to a method for delivering exogenous proteins to the cytosol, by binding a target antigen (such as a protein) to a transport factor that contains a fragment of a bipartite protein exotoxin, but not the corresponding protective antigen. Preferably, the target antigen is fused to the transport factor. Preferred transport factors include the protective antigen binding domain of lethal factor (LFn) from B.anthraxis, consisting of amino acids 1-255, preferably a fragment of at least 80 amino acids that shows at least 80 % homology to LFn, and a fragment of about 105 amino acids from the carboxy portion that does not bind PA. The target antigen can include any molecule for which it would be desirable to elicit a CMI response, including viral antigens and tumor antigens.



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(54) Title: METHODS OF DELIVERY OF EXOGENOUS PROTEINS TO THE CYTOSOL AND USES THEREOF

(57) Abstract: The present invention is directed to a method for delivering exogenous proteins to the cytosol, by binding a target antigen (such as a protein) to a transport factor that contains a fragment of a bipartite protein exotoxin, but not the corresponding protective antigen. Preferably, the target antigen is fused to the transport factor. Preferred transport factors include the protective antigen binding domain of lethal factor (LFn) from *B.anthraxis*, consisting of amino acids 1-255, preferably a fragment of at least 80 amino acids that shows at least 80 % homology to LFn, and a fragment of about 105 amino acids from the carboxy portion that does not bind PA. The target antigen can include any molecule for which it would be desirable to elicit a CMI response, including viral antigens and tumor antigens.

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10 METHODS OF DELIVERY OF EXOGENOUS PROTEINS
 TO THE CYTOSOL AND USES THEREOF

 This invention was supported by National Institutes of Health grant
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15

FIELD OF THE INVENTION

 The present application is directed to a method of delivering
exogenous proteins to the cytosol, novel fusion proteins and uses thereof.

20 BACKGROUND OF THE INVENTION

 Much attention has focused on methods for generating immune
reactions. One class of immune reaction to foreign antigens is the production of
antibodies, typically referred to as humoral immunity. A second form of immune
reaction results from the presentation of antigen by an antigen presenting cell (APC).
25 This type of immune reaction is broadly referred to as cell mediated immunity (CMI),
or T cell responses. Although both types of immune responses are important,
considerable attention has recently focused on CMI. In dealing with infectious
diseases such as AIDS, caused by the Human Immunodeficiency Virus (HIV), the
antibody responses to the virus and portions thereof have not proven sufficient to
30 confer immunity. Similarly, in dealing with exogenous proteins associated with many

5 malignancies, the antibody responses have also not proven sufficient. Thus, speculation has focused on generating CMI responses.

In order to elicit CMI, an antigen must be bound to a major histocompatibility complex (MHC) class I or II molecule on the surface of the APC. The class I molecules typically present antigens externally, such as endogenous
10 proteins, those from viral infections, and tumor antigens. Antigen-specific T cells typically recognize infected target cells when the pathogen-derived (or cancerous) peptide epitopes (usually 8 to 10 amino acids) are presented by molecules encoded by the host class I MHC (7). These epitopes are derived from cytoplasmic proteins cleaved by the proteasome into small peptide fragments. These are then transported
15 into the lumen of the endoplasmic reticulum (ER), where they complex with newly synthesized MHC-I molecules and are subsequently transported to the cell surface, where recognition by T cells occurs (8-13). Antigens in the extracellular fluid (exogenous antigens) generally do not gain access into this processing compartment in most cells. Thus, a significant challenge to eliciting CMI with a vaccine is the
20 delivery of exogenous antigens to the cytosol for presentation by MHC class I molecules. It would be desirable to be able to generate vaccines to a wide variety of infectious diseases, such as HIV, as well as cancers, such as prostate cancer, breast cancer and melanoma.

For example, growing evidence suggests that CMI plays an essential
25 role in controlling HIV infection (Ogg et al., *Science* **279**:2103-6 (1998); Schmitz et al., *Science* **283**:857-60 (1999); Brodie et al., *Nat. Med.* **5**:34-41 (1999)). Individuals who have been exposed to HIV but remain uninfected often have antiviral CMI but no antibody response. The viremia of primary infection resolves as viral specific

5 cytotoxic T lymphocytes (CTL) develop, before the development of specific
antibodies (Letvin, *Science* **280**:1875-96 (1998)). These data illustrate the central role
CMI plays in controlling HIV infection.

Many tumors are associated with the expression of a particular protein
and/or the over-expression of certain proteins. For example, prostate cancer is
10 associated with elevated levels of protein such as Prostate Specific Antigen (PSA).
Breast cancers can be associated with the expression and/or over-expression of
protein such as Her-2, Muc-1, CEA, etc. Thus, considerable attention has been aimed
at trying to generate immune responses, particularly developing CMI, to such antigens
in the treatment of such malignancies.

15 Approaches to developing cell mediated immunity to infectious
diseases have included using the entire infectious agent, for example, by making
genetically engineered inactivated viruses or using a killed infectious agent. Another
approach has been subunit vaccines, which is presenting one or more antigens (but not
the entire virus) to a subject.

20 In order to generate CMI, antigen must be delivered to the interior of
the cell. Exogenous proteins are poorly taken up by the cell. Accordingly, the
preferred method has been using procedures such as viral vectors, liposomes, naked
DNA or a similar approach. However, such approaches have many draw backs. For
example, many recombinant viruses generate antigenic reactions themselves, upon
25 repeated administration. Since standard forms of generating immune reactions
typically require an initial injection, referred to as the prime, and subsequent
injections, referred to as boosts, to achieve a satisfactory immunity, this can be a
serious problem. Moreover, while much attention has been placed on improving the

5 safety of viral vectors, there are always certain risks. For example, many of the target
populations, such as those infected with HIV, may have a weakened immune system.
Thus, certain viral vectors that are perfectly safe in many individuals may pose some
degree of risk to these individuals. Methods of delivering protein to cells have also
not proven entirely satisfactory as of this time. Accordingly, there is a need for new
10 and simple methods to deliver an antigen to the cytosol to stimulate CMI.

In trying to develop CMI responses, there have also been technical
problems with the difficulty in measuring these responses.

Current available laboratory assays to detect cell-mediated immune
responses have serious shortcomings, especially when applied to large vaccine
15 efficacy trials in various clinical settings. This is because the available equipment and
technical support required to measure CMI using current techniques are often minimal
in the setting where they are required, in the field. CTL are thought to play a crucial
role in controlling HIV-1 infection, and many HIV-1 vaccine candidates are designed
to stimulate T cell responses as well as neutralizing antibodies (1-6). However, the
20 standard laboratory methods for detecting CMI, such as HIV-specific CTL, are
complex, time consuming, and often restricted to highly specialized facilities. An
improved method for measuring T cell responses will have a significant effect on the
development of all T cell dependent vaccines or immune therapies. This strategy can
potentially be applicable to other fields of research where CMI responses are known
25 to play an important role in prevention and control of the diseases.

One difficulty in reliably detecting CMI response *in vitro* results from
the unique requirement for antigen presentation. As described above, the delivery of
exogenous proteins to the cytosol for presentation to T cells by MHC class I

5 molecules represents a significant challenge. This physical partition of the class I pathway has been a major barrier to detect T cell responses *in vitro*. Consequently, most of the current laboratory methods in measuring CMI utilize live viral or bacterial vectors to deliver antigens into cytosol, among which recombinant pox such as vaccinia viruses are the most commonly used. Another approach is to externally load
10 MHC-I molecules on surface of target cells with synthetic peptides (10 to 20 amino acids) derived from known CTL epitopes. These methods have serious limitations in general clinical uses. The use of a live viral vector, such as a recombinant vaccinia virus, requires trained and immunized laboratory personnel, including minimum containment facilities as precautionary safety measures. Synthetic peptides are not
15 only prohibitively expensive, but the design of the “universal” peptide profile that fit the diversified MHC-I molecules in various populations is extremely difficult. The challenge for the development of assays to measure T cell responses is, therefore, to deliver large pieces of exogenous antigens into cytosol without resorting to live recombinant viral or bacterial vectors.

20 Accordingly, it would be desirable to have kits that could be used for measuring CMI *in vitro*. It would be particularly desirable to have kits that could be readily used in remote locations such as Africa, India and Asia, where there are many proposals to test a number of vaccine candidates such as vaccines against HIV.

We have now discovered that a family of bipartite protein exotoxins,
25 such as *Bacillus anthracis*, contains fragments that can be used for the delivery of exogenous antigens, such as proteins, to the cytosol. One preferred protein fragment from these proteins is from the N-terminal portion that contains the protective antigen (PA) binding domain, but not those portions resulting in toxicity to the cell. More

5 preferably, that fragment has been modified to remove the specific domain that binds to PA.

B. anthracis is the causative agent of anthrax in animals and humans.

The toxin produced by *B. anthracis* consists of two bipartite protein exotoxins, lethal toxin (LT) and edema toxin. LT is composed of protective antigen (PA) and lethal factor (LF), whereas edema toxin consists of PA and edema factor (EF). None of
10 these three components, PA, LF, and EF, alone is toxic. Once combined however, edema toxin causes edema and LT causes death by systemic shock in animals and humans. Consistent with its critical role in forming both toxins, PA has been identified as the protective component in vaccines against anthrax. The molecular
15 mechanism of anthrax toxin action is currently hypothesized as follows: PA is a 735-amino acid polypeptide that binds to the surface of mammalian cells by cellular receptors. Once bound, PA is activated by proteolytic cleavage by cellular proteases to a 63-kDa molecule capable of forming a ring-shaped heptamer in the plasma membrane of the targeted cell (Figure 1) (6, 7). The PA heptamer then binds either
20 EF or LF, which are internalized by endocytosis. After endosomal acidification, PA enables EF or LF to enter the cytosol, presumably by means of a pore formed by the heptamer. Within the cytosol, EF acts as an adenylate cyclase (8) to convert ATP to cAMP. Abnormally elevated levels of cAMP perturb cellular metabolism.

The action of LF in the cytosol causes the death of host cells by a
25 mechanism that is not well understood. LF induces over-production of a number of lymphokines (9), contributing to lethal systemic shock in host animals. Recent studies also show that LF has two enzymatic activities: it can act as a zinc metalloprotease (10), and it inactivates the mitogen-activated protein kinase (11).

5 Although it is still not clear how these two enzymatic activities of LF are connected, both are required for LF toxicity. It has previously been reported that anthrax toxin B moieties may be used to deliver eptiopes which in turn elicit an antibody response by the immune system, in the presence of PA (WO 97/23236).

LF is a 796-aa polypeptide, and the functional domain for both
10 enzymatic activities is located between amino acids 383 and 796 (Figure 2A) (12). The N-terminal truncated LF without this catalytic domain completely lacks any toxic effect when mixed with PA and added to cultured macrophages or when injected into animals. It does, however, still bind to PA effectively. The PA binding domain of LF (LFn) consists of amino acids 1-255 (Figure 2B) (12).

15

SUMMARY OF THE INVENTION

The present invention provides methods of delivering exogenous antigens to the cytosol, novel fusion proteins, and uses thereof.

We have now further found that one can use a transport factor that is
20 lethal factor modified to inactivate toxin domains, fragments thereof such as LFn and fragments of LFn, such as a fragment containing the carboxy portion of that fragment, as a transport factor, fused to a target antigen, without PA to deliver the antigen to the cytosol. Preferably, the transport factor is LFn or a fragment thereof. One preferred group is LFn fragments do not contain the PA binding domain. More preferably, the
25 transport factor is an LFn fragment. For example, the 60 carboxy most amino acids of LFn can be used as a transport factor; still more preferably the 80 carboxy-most amino acids. One can also use other fragments. For example, one can use fragments

5 containing more of the lethal factor protein as long as one inactivates the toxin
portion. Preferably the transport factor fragment contains a portion of the 80 carboxy-
most amino acid residues of LFn and contains other portions of the fragment as long
as those portions containing toxicity are removed. Preferably the fragment is 350
amino acids or less, still more preferably it is 300 amino acids or less, even more
10 preferably it is 250 amino acids or less. One preferred fragment is 105 amino acids or
less. A more preferred fragment is 80 amino acids or less. This transport factor is
then linked to the antigen you wish to bring to the cytosol. This can be done by
techniques well known in the art. For example, one could prepare fusion proteins
containing the antigen or antigens that one wants to bring to the cytosol of the cell.

15 The preferred methods of the invention are characterized by novel
polypeptides which elicit in treated animals the formation of a cell mediated immune
response.

This invention provides DNA sequences that code for the novel fusion
polypeptides of the invention, recombinant DNA molecules that are characterized by
20 those DNA sequences, unicellular hosts transformed with those DNA sequences and
molecules, and methods of using those sequences, molecules and hosts to produce the
novel polypeptides and CMI immune response to desired antigens this invention.

In another preferred embodiment, this invention provides a
pharmaceutical composition comprising one or more novel fusion peptides of this
25 invention. Such a composition is effective in eliciting cell mediated immune
responses. In one preferred embodiment it can be used as a vaccine. In another
embodiment, these fusion proteins can be used to create producer cells, preferably

5 bacterial producer cells for a variety of proteins, particularly proteins that have proven difficult to express in such cells.

In another preferred embodiment, this invention provides a method for measuring cell mediated immune responses.

In a further preferred embodiment, this invention provides a kit for
10 measuring cell mediated immune responses *in vitro*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a drawing depicting the PA-mediated entry of LFn into a cell via endocytosis, and subsequent presentation by MHC Class I molecules.

15 Figures 2A-C show the amino acid sequence of various lethal fragment (LF) polypeptides (SEQ ID NO:1). Figure 2A shows the full length amino acid sequence of LF. Figure 2B shows the amino acid sequence of the first 289 amino acids of LFn (SEQ ID NO:2). Figure 2C shows the sequence of amino acids 185-289 of lethal factor, sometimes referred to as Fragment 3 (SEQ ID NO:3).

20 Figures 3 A-B are graphs that show that LFn presentation of targets is PA-independent. Well characterized CTL clones were tested for activity in a standard chromium release assay at effector to target (E:T) 10:1. Targets were HLA-matched EBV-transformed cell lines that were sensitized overnight with LFN in the presence or absence of PA. Controls used were recombinant vaccinia vectors
25 (NYCBH for control and rVV-Nef or Env peptide for appropriate clone). Figure 3A shows activity of a Nef-specific clone (KM) was tested against B60 restricted target cells incubated overnight with Nef recombinant vaccinia vectors, PA and LFnNef or

5 LFn Nef alone. Background/control for each target was included (control, PA-LFn, LFn). Figure 3B shows Env-specific CTL clone (SP 511) recognizes targets labeled with LFnEnv in a dose dependent fashion and was again PA-independent. Control used were peptide Env 106B(100 μ g/ml). Dose of LFn construct is listed as μ g/ml (LfnEnv 5 = 5 μ /ml of LfnEnv).

10 Figures 4 A-B are graphs that show that LFn-HIV recognition is HLA-restricted. 4A shows HLA-B14 restricted Gag clone (AC13) was tested for activity using HLA matched and mismatched B-LCL sensitized with LFn-p24 and tested in standard chromium release assay. Rvv-Gag was used as control. 2B shows HLA-restriction for B60 Nef clone (KM) was demonstrated using HLA matched and
15 mismatched targets sensitized with LFnNef. TAP-deficient (T2), HLA-B60 target cells were sensitized with LFn-Nef and the optimal Nef peptide and tested for lysis. Lack of activity with T2 LFnNef target cells suggests requirement for proteasome processing. All clones were used at E:T 10:1 and lysis level subtracted from those of background controls. NYCBH was used for recombinant vaccinia virus control and
20 LFn alone for LFnP24 and LFnNef. Positive control included Nef peptide 180 and recombinant vaccinia vector Gag (rvv-Gag).

 Figures 5 A-B are graphs that show the internalization and processing of LFn-HIV. 5A shows Nef-specific clone (KM) lysis of HLA-matched target cells sensitized with LFnNef in the presence of brefeldin A, cytochalasin B and
25 chloroquine compared to LFnNef alone. Co-incubation with cytochalasin B and brefeldin A abrogated the recognition by a Nef-specific clone but not with chloroquine. 5B shows activity of the same Nef clone with target infected overnight with recombinant vaccinia virus expressing Nef or sensitized with the optimal epitope

5 peptide Nef 180 alone or in the presence of chloroquine, cytochalasin B or brefeldin A. No significant decrease in activity was seen when target cells and CTL clone were incubated in the presence of cytochalasin B, chloroquine or brefeldin A. Lysis levels shown are subtracted from background lysis which were less than 10%.

Figures 6A-E are graphs that show LFn-HIV expression in the Elispot
10 assay. Cryopreserved PBMCs from HIV-1 infected individuals with known HIV-specific CTL activity or epitopes were used in the Elispot assay using recombinant vaccinia viruses, or LFn-HIV. LFn alone and NYCBH were used as control. Duplicate wells were used at 100K cell/well. Results are reported as SFC/million (spot forming cells per million).

15 Figures 7 A-B are FACS plots that show LFn-HIV expression in intracellular flow-based assay. Intracellular production of IFN- γ in Nef-specific clone (KM)(A) as well as from PBMC of a seropositive individual (AC2) with Env-specific CTL activity (B). Unstimulated cells were used as negative control (1) and recombinant vaccinia viruses as positive control (2) LfnNef and LfnEnv were used
20 respectively (3).

Figures 8A and B are drawings depicting LFn fusion proteins.

Figure 9 is a series of drawings depicting LFn fusion proteins.

Figure 10 shows cellular uptake of LFn-GFP into CHO cells. CHO cells were cultured in collagen treated chamber slides to reach 80% confluence and
25 were then incubated with purified LFn-GFP for one hour, followed by washing extensively with PBS and PBS plus proteases to remove membrane bound proteins. The cells were then stained with the anti-transferrin antibodies and fixed with

5 paraformaldehyde. The slides were examined by confocal microscopy for green color (GFP, shown in Figure 10 A-D) and red color (anti-transferrin, shown in Figure 10 E-H), respectively. A third image is presented for each field by super-expose the green image with the red image of the same cells (Figure 10 I-L). Consequently, a yellow spot indicates that the GFP may be in the same spot with the transferrin protein, thus
10 providing a reference to the location of GFP inside the cells. As shown in Figure 10A-D, a significant number of green spots were visible in cells treated with LFn-GFP for one hour, suggesting the LFn-GFP can indeed enter into cells.

Figure 11 shows cellular uptake of GFP into CHO cells, using conditions as described for Figure 10. Figure 11 A, D, and G show the results of
15 incubating CHO cells with GFP for 1 hour; Figure 11 B-C, E-F, and H-I show the results of incubating CHO cells with GFP for two hours. Figure 11 A-C show fields of cells examined by confocal microscopy for green color (GFP); Figure 11 D-F show the corresponding fields examined by confocal microscopy for red color (anti-transferrin), respectively. A third image is presented for each field by super-exposing
20 the green image with the red image of the same cells (Figure 11 G-I). GFP-treated cells showed few green spots (Figure 11).

Figure 12 shows co-localization of GFP and LAMP-2 immunofluorescence to indicate the lysosome, using the identical experimental procedures described above for Figure 10. Figures 12 A-D show fields of cells
25 examined by confocal microscopy for red color (LAMP-2); Figures 12 E-H show the corresponding fields examined by confocal microscopy for green color (LN-GFP). A third image is presented for each field by super-exposing the green image with the red image of the same cells (Figures 12 I-L).

5 Figure 13 shows co-localization of GFP and immunofluorescence to
EEA-1 to indicate the endosome, using the identical experimental procedures
described above for Figure 10. Figures 13 A-D show fields of cells examined by
confocal microscopy for red color (EEA-1); Figures 13 E-H show the corresponding
fields examined by confocal microscopy for green color (LN-GFP). A third image is
10 presented for each field by super-exposing the green image with the red image of the
same cells (Figures 13 I-L).

 Figure 14 shows co-localization of GFP and an anti-Golgi antibody,
using the identical experimental procedures described above for Figure 10. Figures
14 A-B show fields of cells examined by confocal microscopy for red color (anti-
15 Golgi antibody); Figures 14 C-D show the corresponding fields examined by confocal
microscopy for green color (LN-GFP). A third image is presented for each field by
super-exposing the green image with the red image of the same cells (Figures 14 E-F).

 Figure 15 shows co-localization of GFP and anti-20s
immunofluorescence to indicate the proteosome. Conditions were similar to those
20 used in Figure 10 except HeLa cells were uncubated with 40 µg/ml LN-gfp for 2
hours. Figure 15 A-D show fields of cells examined by confocal microscopy for red
color (anti-20s antibody); Figure 15 E-H show the corresponding fields examined by
confocal microbscopy for green color (LN-GFP), respectively. A third image is
presented for each field by super-exposing the green image with the red image of the
25 same cells (Figure 15I-L). By comparing the different images in Figures 12 – 15, the
super-exposed images that have the most yellow spots are those shown in Figure 15 in
which the green spots representing the intracellular LFn-GFP overlap significantly
with the red spots representing cellular proteosome.

5 Figure 16 shows that adding PA did not increase the number of green spots inside cells in comparison with those in the absence of PA, under conditions similar to those used in Figure 15. Figures 16 A-F show incubation of HeLa cells with LN-GFP in the absence of PA; Figures 16 G-L show incubation of HeLa cells in the presence of PA. Figures 16 A-B and G-H show fields of cells examined by
10 confocal microscopy for red color (anti-20s antibody); Figures 16 C-D and I-J show the corresponding fields examined by confocal microscopy for green color (LN-GFP), respectively. A third image is presented for each field by super-exposing the green image with the red image of the same cells (Figures 16 E-F and K-L).

 Figure 17 Three groups of BALB/c mice, four in each group, were
15 immunized i.p. with different antigen formulations, respectively (15 μ g LFn-p24 plus 4 μ g PA, 15 μ g LFn-p24 plus 4 μ g PA plus Alum, 15 LFn-p24 only plus Alum). At one-week post the immunization, splenic mononuclear cells from immunized BALB/c mice were used as the source of CTL. The CTL in the splenic cultures were activated in vitro by culturing with gamma-irradiated and peptide-pulsed BALB/c splenocytes
20 from un-immunized animals. After 6 days of culture in a 37°C CO₂ incubator, mature CTL (effector cells) were tested for their ability to lyse either ⁵¹Cr-labeled P20 peptide-pulsed P815 cells (positive targets) or ⁵¹Cr-labeled medium-pulsed cells (negative targets). The percent lysis shown were subtracted from the background lysis of negative targets and presented as the average of each group.

25 Figure 18 Three groups of BALB/c mice, four in each group, were immunized i.p. with 15 ug LFn-p24, 15 ug MLFn-p24, and 15 ug p24, respectively. The CTL in splenic tissues were tested one after the immunization.

5

DETAILED DESCRIPTION OF THE INVENTION

We have now discovered a method for delivering exogenous proteins to the cytosol, by binding a target antigen (such as a protein) to a transport factor that contains a fragment of a bipartite protein exotoxin in the absence of a protective antigen (PA). Preferably, the target antigen is fused to the transport factor.

Preferably, the transport factor is the protective antigen binding domain of lethal factor from *B. anthracis*, consisting of amino acids 1-288 (SEQ ID NO:2) or a fragment thereof that does not contain the PA binding domain such as the carboxy portion. For example, a fragment of about the 80 carboxy-most amino acids of preferred group of amino acid LFn 1 amino acid fragments for the transport factor comprises at least 80 amino acids that show at least 80% homology to SEQ ID NO: 2 using Blast on default settings. Still more preferably, the fragment has at least 90% homology thereto, even more preferably, it has at least 95% homology thereto. Preferably, that fragment does not contain the PA binding domain. A preferred transport factor is the LFn fragment or a portion thereof. More preferably, the transport factor comprises at least the 60 carboxy-most amino acids of LFn but not the PA binding domain. One preferred fragment has about 105 amino acids or less from the carboxy portion of SEQ ID NO:2.

The target antigen can include any molecule for which it would be desirable to elicit a CMI response, including viral antigens and tumor antigens.

One embodiment of the invention provides a composition including the novel fusion polypeptide for generating. Another embodiment of the invention

5 includes assays for measuring CMI responses by delivering exogenous proteins to the cytosol. A preferred embodiment provides kits for measuring CMI responses.

Novel fusion polypeptides of the present invention comprise a transport factor linked to a target antigen. The transport factor can include a fragment of any bipartite protein exotoxin that delivers the protein to the cytosol in the absence of protective antigen without including any fragments that are toxic. This transport
10 method is independent of the PA associated pathway and thus PA is unnecessary. A preferred exotoxin is Lethal Factor (LF) of *B. anthracis* (SEQ ID NO:1) (Figure 2A). The PA binding domain of LF, is part of LFn, or a fragment thereof that can transverse a cell membrane consists of amino acids 1-255 (SEQ ID NO:2) (Figure
15 2B). Any fragment of LFn can be used as the transport factor. Preferably it does not contain the PA binding domain, which is present in the N-terminal half of LFn (aa 1-149). One preferred fragment has 105 amino acids or less from the carboxy portion of SEQ ID NO:2. Preferably, the 60 carboxy-most amino acids, still more preferably the 80 carboxy-most amino acids. A preferred transport factor is Fragment 3 (SEQ ID
20 NO:3) (Figure 2C).

One can also use other fragments as the transport factor. Preferably, the transport factor is 350 amino acids or less, still more preferably, 300 amino acids or less, even more preferably, it is 250 amino acids or less.

Other fragments preferred as the transport factor have at least 55%
25 homology to Fragment 3 of LFn (SEQ ID NO:3) (Figure 2C). For example, a fragment of Edema Factor of *B. anthracis* has approximately 57% homology to Fragment 3_g using Blast on default settings. More preferably, it has at least 65% homology thereof; still more preferably, it has at least 75% homology thereto; even

- 5 more preferably, it has at least 80% homology thereto; even more preferably, it has at least 90% homology thereto; even more preferably, it has at least 95% homology thereto.

The transport factor is linked to any antigen whose delivery to the cytosol is desired. Preferably, the linkage is in the form of a fusion protein.

- 10 However, other linkers known in the art can be created. For example, a linker unit can be part of the transporter that can then be chemically linked to the target antigen. Preferred antigens include viral, bacterial, parasitic, and tumor associated antigens. Preferred viral antigens include proteins from any virus where a cell-mediated immune response is desired. Particularly preferred viruses include HIV-1, HIV-2, 15 hepatitis viruses (including hepatitis B and C), Ebola virus, West Nile virus, and herpes virus such as HSV-2. Preferred bacterial antigens include those from *S. typhi* and *Mycobacteria* (including *M. tuberculosis*). Preferred parasitic antigens include those from *Plasmodium* (including *P. falciparum*).

- Preferred tumor antigens include those epitopes which are recognized 20 in eliciting T cell responses, including but not limited to the following: prostate cancer antigens (such as PSA, PSMA, etc.), breast cancer antigens (such as HER2/neu, mini-MUC, MUC-1, HER2 receptor, mammoglobin, labyrinthine, SCP-1, NY-ESO-1, SSX-2, N-terminal blocked soluble cytokeratin, 43 kD human cancer antigens, PRAT, TUAN, Lb antigen, carcinoembryonic antigen, polyadenylate 25 polymerase, p53, mdm-2, p21, CA15-3, oncoprotein 18/stathmin, and human glandular kallikrein), melanoma antigens, and the like.

Preferably, when one is trying to generate an immune response in a subject, an immune adjuvant is also used. Adjuvants are known in the art and include

5 cytokines such as IL-2, Ig-IL-2, CM-CSF, CpG, RIBL Detox (Ribi
Immunochemical), QS21 (Cambridge Biotech), incomplete Freund's adjuvant and
others. We have unexpectedly found that although Alum can actually inhibit CTL
induction, in the present system, Alum is preferred for stimulating specific CTL.

The methods of the present invention can also be used to identify
10 additional cancer antigens, by making libraries of tumor antigens fused to the
transport factor in conjunction with the CMI assays, described below.

The target antigen can be of any size that allows delivery to the
cytosol. Preferably, the target antigen is less than 750 amino acids, still more
preferably, less than 600 amino acids, even more preferably, less than 500 amino
15 acids.

The novel fusion polypeptides of the present invention can include a
single target antigen or multiple target antigens as part of a single fusion protein. A
preferred fusion polypeptide includes fragments of several HIV-1 proteins, such as
gag and nef (Figures 8, 9).

20 One can also create epitopes using multiple strains of an infectious
virus such as with HIV. (See Figure 9).

The novel fusion polypeptides may be part of larger multimeric
molecules which may be produced recombinantly or may be synthesized chemically.
Such multimers may also include the polypeptides fused or coupled to moieties other
25 than amino acids, including lipids and carbohydrates.

5 Preferably, the multimeric proteins consist of multiple T cell epitopes repeated within the same molecule, either randomly, or with spacers (amino acid or otherwise) between them.

 DNA sequences encoding these novel fusion proteins can readily be made. For example, the sequence encoding LFn is well known and can be modified
10 by known techniques, such as deleting the undesired regions, such as variable loops, and to insert any additional desired coding sequences, such linker segments. The sequences encoding various target antigens are also known in the art. In addition, the codons for the various amino acid residues are known and one can readily prepare alternative coding sequences by standard techniques.

15 DNA sequences can be used in a range of animals to express the novel fusion protein, which can then be used for a variety of uses, including in a vaccine composition and in CMI assays, as described below.

 DNA sequences encoding the novel fusion protein can be expressed in a wide variety of host/vector combinations. Vectors include chemical conjugates such
20 as those described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vectors (e.g. a DNA or RNA viral vector), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

 Useful expression vectors for eukaryotic hosts, include, for example,
25 vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus, adeno-associated virus, cytomegalovirus and retroviruses. Useful expression vectors for bacterial hosts include bacterial plasmids, such as those from E. coli, including pBluescript, pGEX-2T, pUC vectors, col E1, pCR1, pBR322, pMB9

5 and their derivatives, wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g. .lambda.GT10 and .lambda.GT11, and other phages. Useful expression vectors for yeast cells include the 2 micron. plasmid and derivatives thereof. Useful vectors for insect cells include pVL 941.

Preferred vectors include viral vectors, fusion proteins and chemical
10 conjugates. Retroviral vectors include Moloney murine leukemia viruses and HIV-based viruses. One preferred HIV-based viral vector comprises at least two vectors wherein the *gag* and *pol* genes are from an HIV genome and the *env* gene is from another virus. DNA viral vectors are preferred. These vectors include herpes virus vectors such as a herpes simplex I virus (HSV) vector (Geller, A. I. *et al.*, *J. Neurochem* 64: 487, 1995; Lim, F. *et al.*, in *DNA Cloning: Mammalian Systems*, D. Glover, Ed., Oxford Univ. Press, Oxford England, 1999); Geller, A. I., *Proc. Natl. Acad. Sci. USA* 90: 7603, 1993; Geller, A. I., *Proc Natl. Acad. Sci USA* 87: 1149, 1990), adenovirus vectors (LeGal LaSalle *et al.*, *Science* 259: 988, 1993; Davidson, *et al.*, *Nat. Genet* 3: 219, 1993; Yang, *et al.*, *J. Virol.* 69: 2004, 1995), and adeno-
15 associated virus vectors (Kaplitt, M.G., *et al.*, *Nat. Genet.* 8:148, 1994). The DNA sequence is operably linked to a promoter that permits expression in the host cell. Such promoters are well known in the art and can readily be selected.

A wide variety of unicellular host cells are useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic
25 and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi, yeast, insect cells such as *Spodoptera frugiperda* (SF9), animal cells such as CHO and mouse cells, African green monkey cells such as COS 1, COS 7, BSC 1, BSC 40, and BMT 10, and human cells, as well as plant cells in tissue

5 culture. The molecules of the present invention can be used to create a range of producer cells . This is particularly useful with certain proteins that are currently difficult to express in cells in large amounts.

The molecules comprising the novel fusion polypeptides encoded by the DNA sequences of this invention may be isolated from the fermentation or cell
10 culture and purified using any of a variety of conventional methods including: liquid chromatography such as normal or reversed phase, using HPLC, FPLC and the like; affinity chromatography (such as with inorganic ligands or monoclonal antibodies); size exclusion chromatography; immobilized metal chelate chromatography; gel electrophoresis; and the like. One of skill in the art may select the most appropriate
15 isolation and purification techniques without departing from the scope of this invention.

Stabilized forms of these novel fusion proteins can readily be made, for example, by conjugates such as a poly(alkylene oxide) conjugate. The conjugate is preferably formed by covalently bonding the hydroxyl terminals of the poly(alkylene
20 oxide) and a free amino group in a portion of the fusion protein that will not affect its conformation. Other art recognized methods of conjugating these materials include amide or ester linkages. Covalent linkage as well as non-covalent conjugation such as lipophilic or hydrophilic interactions can be used.

The conjugate can be comprised of non-antigenic polymeric substances
25 such as dextran, polyvinyl pyrrolidones, polysaccharides, starches, polyvinyl alcohols, polyacryl amides or other similar substantially non-immunogenic polymers. Polyethylene glycol(PEG) is preferred. Other poly(alkylenes oxides) include monomethoxy-polyethylene glycol polypropylene glycol, block copolymers of

5 polyethylene glycol, and polypropylene glycol and the like. The polymers can also be distally capped with C1-4 alkyls instead of monomethoxy groups. The poly(alkylene oxides) used must be soluble in liquid at room temperature. Thus, they preferably have a molecular weight from about 200 to about 20,000 daltons, more preferably about 2,000 to about 10,000 and still more preferably about 5,000.

10 Those of ordinary skill in the art will recognize that a large variety of possible moieties can be coupled to the resultant novel fusion proteins of the invention. See, for example, "Conjugate Vaccines", *Contributions to Microbiology and Immunology*, J. M. Cruse and R. E. Lewis, Jr (eds.), Carger Press, New York, 1989, the entire contents of which are incorporated herein by reference.

15 Coupling may be accomplished by any chemical reaction that will bind the two molecules so long as the novel fusion protein and the other moiety retain their respective activities. This linkage can include many chemical mechanisms, for instance covalent binding, affinity binding, intercalation, coordinate binding and complexation. The preferred binding is, however, covalent binding. Covalent
20 binding can be achieved either by direct condensation of existing side chains or by the incorporation of external bridging molecules. Many bivalent or polyvalent linking agents are useful in coupling protein molecules, such as the antibodies of the present invention, to other molecules. For example, representative coupling agents can include organic compounds such as thioesters, carbodiimides, succinimide esters,
25 disocyanates, glutaraldehydes, diazobenzenes and hexamethylene diamines. This listing is not intended to be exhaustive of the various classes of coupling agents known in the art but, rather, is exemplary of the more common coupling agents (see

- 5 Killen and Lindstrom, *J. Immunol.* 133:1335-2549, 1984; Jansen, F. K., *et al.*, *Imm. Rev.* 62:185-216, 1982; and Vitetta *et al.*, *supra*).

- Preferred linkers are described in the literature. See, for example, Ramakrishnan, S., *et al.*, *Cancer Res.* 44: 201-208 (1984), describing the use of MBS (M-maleimidobenzoyl-N-hydroxysuccinimide ester). See also Umemoto *et al.*, U.S.
- 10 Patent 5,030,719, describing the use of a halogenated acetyl hydrazide derivative coupled to an antibody by way of an oligopeptide linker. Particularly preferred linkers include: (i) EDC (1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride; (ii) SMPT (4-succinimidylloxycarbonyl-alpha-methyl-alpha-(2-pyridyl-dithio)-toluene (Pierce Chem. Co., Cat. (21558G); (iii) SPDP (succinimidyl-6
- 15 [3-(2-pyridyldithio) propionamido] hexanoate (Pierce Chem. Co., Cat #21651G); (iv) Sulfo-LC-SPDP (sulfosuccinimidyl 6 [3-(2-pyridyldithio)-propionamide] hexanoate (Pierce Chem. Co. Cat. #2165-G); and (v) sulfo-NHS (N-hydroxysulfo-succinimide: Pierce Chem. Co., Cat. #24510) conjugated to EDC.

- The linkers described above contain components that have different
- 20 attributes, thus leading to conjugates with differing physio-chemical properties. For example, sulfo-NHS esters of alkyl carboxylates are more stable than sulfo-NHS esters of aromatic carboxylates. NHS-ester containing linkers are less soluble than sulfo-NHS esters. Further, the linker SMPT contains a sterically hindered disulfide bond, and can form conjugates with increased stability. Disulfide linkages, are in
- 25 general, less stable than other linkages because the disulfide linkage is cleaved in vitro, resulting in less conjugate available. Sulfo-NHS, in particular, can enhance the stability of carbodimide couplings. Carbodimide couplings (such as EDC) when used

5 in conjunction with sulfo-NHS, forms esters that are more resistant to hydrolysis than the carbodiimide coupling reaction alone.

The novel fusion proteins of the present invention can be used for the stable expression of proteins. For example, certain proteins are difficult to express in certain expression systems, including bacterial expression systems. Fusion to a
10 transport factor such as LFn can stabilize certain such proteins. We have found that the transport factor is preferably 250 amino acids or less, still more preferably 150 amino acids or less, more preferably 105 amino acids or less, even more preferably 80 amino acids or less.

The novel fusion proteins of the present invention can be used to
15 generate an immune response. For example, as a vaccine.

An exemplary pharmaceutical composition is a therapeutically effective amount of a novel fusion protein that can induce an immune reaction, thereby acting as a prophylactic immunogen, optionally included in a pharmaceutically-acceptable and compatible carrier. The term “pharmaceutically-
20 acceptable and compatible carrier” as used herein, and described more fully below, includes (i) one or more compatible solid or liquid filler diluents or encapsulating substances that are suitable for administration to a human or other animal, and/or (ii) a system, capable of delivering the molecule to a target cell. In the present invention, the term “carrier” thus denotes an organic or inorganic ingredient, natural or synthetic,
25 with which the molecules of the invention are combined to facilitate application. The term “therapeutically-effective amount” is that amount of the present pharmaceutical composition which produces a desired result or exerts a desired influence on the particular condition being treated. For example, the amount necessary to raise an

5 immune reaction to provide prophylactic protection. Typically when the composition is being used as a prophylactic immunogen at least one "boost" will be administered at a periodic interval after the initial administration. Various concentrations may be used in preparing compositions incorporating the same ingredient to provide for variations in the age of the patient to be treated, the severity of the condition, the
10 duration of the treatment and the mode of administration.

In a preferred embodiment, the novel fusion polypeptides of this invention which are also immunogenic polypeptides are incorporated into a multicomponent vaccine which can comprise other immunogenic polypeptides. A multicomponent vaccine may contain novel fusion protein(s) of the present invention
15 to elicit T cell responses, as well as other antigens to elicit B cell responses.

In one preferred method of immunization one would prime with one novel fusion protein and then boost with a different novel fusion protein.

One can also use cocktails containing a variety of different novel fusion proteins to prime and boost with either a variety of different novel fusion
20 proteins or with fusion proteins that contain multiple antigens (Figures 8, 9).

The novel fusion proteins can be used to generate a range of T cells that recognize and interact with a diverse range of antigens, for example, from different HIV strains. The DNA sequence encoding the novel fusion proteins can also be used as a subunit vaccine.

25 In trying to generate an immune reaction such as with a vaccine composition, an adjuvant is preferably also used. Adjuvants include but are not limited to Alum, RIBI Detox (Ribi Immunochemical), QS21 (Cambridge Biotech),

5 and incomplete Freund's adjuvant. Alum is a preferred adjuvant. Another group of
adjuvants include immune stimulators such as cytokines such as IL-12, IL-4 and
costimulatory molecules such as B7. A wide range of molecules having immune
stimulating effects are known including accessory molecules such as ICAM and LFA.
In a preferred embodiment GM-CSF is administered to the patient before the initial
10 immune administration. GM-CSF may be administered using a viral vector or an
isolated protein in a pharmaceutical formulation. Combinations of adjuvants can be
used such as GM-CSF, ICAM and LFA. While a strong immune response is
typically generated to infectious disease antigens, tumor associated antigens typically
generate a weaker immune response. Thus, immune stimulators such as described
15 above are preferably used with them. As aforesaid, Alum is a preferred adjuvant.

The immune stimulatory composition of the present invention may be
used advantageously with other treatment regimens. For example, the system may be
used in conjunction with traditional treatment options for cancer including surgery,
radiation therapy, chemotherapy and hormone therapy. For example, a breast cancer
20 vaccine comprising a novel fusion protein of the present invention can be used in
conjunction with tamoxifen citrate, which interferes with the activity of estrogen. The
system may also be combined with immunotherapy, e.g. using HerceptinTM
(trastuzumab), an anti-HER2 humanized monoclonal antibody developed to block the
HER2 receptor; bone marrow transplantation; and peripheral blood stem cell therapy
25 can also be used. Other preferred treatment regimens to be used in conjunction with
the present composition include angiogenesis inhibitors and cytotoxic agents.

The term "compatible", as used herein, means that the components of
the pharmaceutical compositions are capable of being commingled with a small

5 molecule, nucleic acid and/or polypeptides of the present invention, and with each other, in a manner such that does not substantially impair the desired pharmaceutical efficacy.

Doses of the pharmaceutical compositions of the invention will vary depending on the subject and upon the particular route of administration used.

10 Dosages can range from 0.1 to 100,000 $\mu\text{g/kg}$ per day, more preferably 1 to 10,000 $\mu\text{g/kg}$. Preferred doses of the compositions are preferably at least 2 $\mu\text{g/ml}$. By way of an example only, an overall dose range of from about, for example, 1 microgram to about 300 micrograms might be used for human use. This dose can be delivered at periodic intervals based upon the composition. For example on at least two separate
15 occasions, preferably spaced apart by about 4 weeks. Other compounds might be administered daily. Pharmaceutical compositions of the present invention can also be administered to a subject according to a variety of other, well-characterized protocols. For example, certain currently accepted immunization regimens can include the following: (i) administration times are a first dose at elected date; a second dose at 1
20 month after first dose; and a third dose at a subsequent date, e.g., 5 months after second dose. See Product Information, *Physician's Desk Reference*, Merck Sharp & Dohme (1990), at 1442-43. (e.g., Hepatitis B Vaccine-type protocol); (ii) for example with other vaccines the recommended administration for children is first dose at elected date (at age 6 weeks old or older); a second dose at 4-8 weeks after first dose;
25 a third dose at 4-8 weeks after second dose; a fourth dose at 6-12 months after third dose; a fifth dose at age 4-6 years old; and additional boosters every 10 years after last dose. See Product Information, *Physician's Desk Reference*, Merck Sharp & Dohme (1990), at 879 (e.g., Diphtheria, Tetanus and Pertussis-type vaccine protocols).

5 Desired time intervals for delivery of multiple doses of a particular composition can be determined by one of ordinary skill in the art employing no more than routine experimentation.

 The novel fusion proteins of the invention may also be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in
10 medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof and are not excluded from the scope of this invention. Such pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic,
15 acetic, salicylic, p-toluene-sulfonic, tartaric, citric, methanesulfonic, formic, malonic, succinic, naphthalene-2-sulfonic, and benzenesulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group. Thus, the present invention also provides pharmaceutical compositions, for medical use, which
20 comprise nucleic acid and/or polypeptides of the invention together with one or more pharmaceutically acceptable carriers thereof and optionally any other therapeutic ingredients.

 The compositions include those suitable for oral, rectal, intravaginal, topical, nasal, ophthalmic or parenteral administration, all of which may be used as
25 routes of administration using the materials of the present invention. Other suitable routes of administration include intrathecal administration directly into spinal fluid (CSF), direct injection onto an arterial surface and intraparenchymal injection directly into targeted areas of an organ. Compositions suitable for parenteral administration

5 are preferred. The term "parenteral" includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. Intramuscular administration is preferred.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

10 Methods typically include the step of bringing the active ingredients of the invention into association with a carrier which constitutes one or more accessory ingredients.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the nucleic acid and/or polypeptide of the
15 invention in liposomes or as a suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, or an emulsion.

Preferred compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the molecule of the invention which is preferably isotonic with the blood of the recipient. This aqueous preparation
20 may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic
25 sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

5 To further improve the likelihood of producing a cell mediated
response as provided by the invention, the amino acid sequence of polypeptides
encoded by a nucleotide sequence of the present invention may be analyzed in order
to identify desired portions of amino acid sequence which may be associated with
receptor binding. For example, polypeptide sequences may be subjected to computer
10 analysis to identify such sites.

Complexes that form with molecules of the present invention can be
detected by appropriate assays, such as the CMI assays discussed below and by other
conventional types of immunoassays.

The present invention provides assays to measure cell-mediated
15 immune responses *in vitro*. Current available laboratory assays to detect CMI
responses have serious shortcomings, especially when applied to large vaccine
efficacy trials at various clinical settings. This is because the available equipment and
technical support are minimal. As discussed above, the belief that CTL play a crucial
role in controlling HIV-1 infection, many HIV-1 vaccine candidates are designed to
20 stimulate T cell responses as well as neutralizing antibodies (1-6).

The method of the present invention for measuring T cell responses
will have a significant effect on the development of all T cell dependent vaccines or
immune therapies. This strategy is applicable to other field of research where CMI
responses play an important role in prevention and controlling of the diseases.

25 In a preferred embodiment, the present invention provides use of the
novel fusion polypeptides in the Elispot assay. The main advantage of the Elispot
assay is that a large number of CTL responses can be evaluated effectively and
efficiently in a large number of people (see for example, Lalvani et al., *J. Exp. Med.*

5 **186:859-65** (1997)). The Elispot assay can use any standard cells, including cryopreserved PBMCs as well as CD8⁺ CTL cells. For example, CD8⁺ CTL clones from HIV-1 positive individuals.

Another preferred embodiment provides use of the novel fusion polypeptide in flow-based intracellular cytokine assays. Flow cytometry-based assays
10 detect the intracellular accumulation of cytokines after *in vitro* stimulation of antigen specific T cells. CTLs are stimulated with antigens and incubated with brefeldin A, which inhibits protein transport and allows intracellular accumulation of newly synthesized proteins such as cytokines. Surface markers (CD8, CD3) and intracellular staining of IFN- γ and CD69 allows detection and quantitation of specific
15 cell populations against antigenic epitopes in the context of uncharacterized MHC backgrounds.

The CMI assays using the novel fusion polypeptides of the present invention can be used to evaluate cellular immune responses against a broad array of viral, bacterial, and tumor antigens. The assays can be used in either evaluating
20 vaccine efficacy or in arresting the statues of infection by infectious pathogens.

The CMI assays using the novel fusion polypeptides of the present invention can also be used as early diagnostic kits to detect cancer antigens.

The present invention also provides a kit for detecting CMI responses. One preferred kit contains novel fusion proteins in these tubes, wells, etc. The kit
25 preferably contains viral or tumor containing reagents to detect CMI responses *in vitro*. Preferably, the kit contains an apparatus for removing a biological specimen such as blood. In a preferred embodiment, the kit will contain instructions for

5 detecting CMI responses. Preferably, the kit contains the novel fusion protein in a lyophilized form.

A preferred kit includes the novel fusion protein lyophilized and coated onto the inside of a tube used for the collection of blood. This allows blood to be drawn from an individual directly into the tube, incubated for several hours (for
10 example, 4 hours), and then fixed. Such fixed samples are stable for use in CMI assays for extended periods of time (for example, two weeks). The kit is particularly useful for correcting samples in the field, for example, during vaccine efficacy trials, which typically occur in remote locations. Similarly, other preferred embodiments include the lyophilized protein coated onto any surface, such as a dish, a well, or a
15 tube, which is amenable to adding a small volume of a biological specimen. Preferred biological specimens include blood, urine, sputum, stool, supernatant from cerebrospinal fluid, and cell samples.

In a specific example, LFn fusion proteins such as those described above which can contain only the carboxy portion of LFn, can in the absence of PA
20 sensitize CTL target cells in a MHC-I restricted manner. In this novel method of presenting exogenous protein antigens to the MHC-I pathway, the cytosolic delivery of the antigens relies on the functional transportation associated with intracellular antigen processing, without resorting to live viral or bacterial vectors. These LFn fragments are an example of a new class of proteins that enters the MHC-I pathway
25 from the cytosol. The fact that for example, LFn can deliver HIV-1 antigens into cytosol in the absence of PA is in contrary to the current hypothesis of how anthrax lethal toxin works (32). In the normal case, LF (anthrax lethal factor) is dependent on

5 PA to exert its toxic effect and possibly the toxic domain of LF possess unknown functions that prevent cell entry.

Additionally, fusion proteins such as LFn-HIV can be used as a tool to refine and simplify the methods for detecting T cell responses in vaccine trials. This method is also useful for studies of other intracellular viral or bacterial diseases, such
10 as viral hepatitis, TB, and malaria. The present invention can circumvent the need for using live recombinant vaccinia viruses in the current CMI assays. It also carries several advantages over using overlapping synthetic peptides, which are not only expensive, but that their theoretical coverage of different populations with HLA-I diversity remains unproven. The novel antigen delivery provides simplified CMI
15 assays that are widely applicable to field use. Furthermore, these LFn fusion proteins are easily produced in E.coli, purified in relatively simple procedures, and are stable.

All of the novel fusion polypeptides provided by this invention, and the DNA sequences encoding them, are substantially free of a *B. anthracis* protective antigen or fragments thereof, and thus may be safely used in a variety of applications
20 without the risk of unintentional damage to the host. Accordingly, the novel fusion polypeptides of this invention are particularly advantageous in compositions and methods for generating a broad range of cell-mediated responses to target antigens.

The following Examples serve to illustrate the present invention, and are not intended to limit the invention in any manner.

25

EXAMPLE 1

Materials and Methods

5 LFn-HIV fusion proteins

The DNA fragment encoding env gp120, gag p24 from HIV-HXB were amplified by PCR, cloned into the LFn expression plasmid pET15bLFn, and sequenced to verify the inframe fusion between the LFn and the HIV coding sequence. The Nef coding sequence was amplified from HIV-ELI. The protein expression vector for LFn and its fusion derivatives is the pET15b plasmid (Novagen; Madison, WI). The main features of this vector system include an inducible T7 promoter, an internal His-Tag for protein purification, and multiple cloning sites. The recombinant LFn is expressed in *E. coli* as an intracellular soluble protein with 6 tandem histidine residues at its N-terminal end (14). Bacteria were grown in a ten-liter Bioflow 2000 bench top bioreactor (New Brunswick Scientific, NJ). Purification of the His-tagged proteins was conducted using a commercial kit according to the manufacturer's protocol (Novagen). Fragments of LFn can be made by modifying the LFn coding region by known techniques.

Reagents

20 Synthetic peptides were synthesized on an Applied Biosystems Peptide Synthesizer (Model 430A). Recombinant vaccinia viruses used were vAbT 141 (Gag), vAbT 299 (Env), and Nef (15) with NYCH as control vaccinia vector. Brefeldin A, cytochalasin B and chloroquine (Sigma, St Louis, MO) were added to cell culture for 2 hours and washed twice before adding back to the cells. Antibodies for flow analysis were obtained from Becton-Dickinson (San Jose, CA)

Flow-based intracellular cytokine staining

5 Cryopreserved PBMCs were incubated overnight with LFN-HIV
(30ug/ml), recombinant vaccinia vectors (MOI 3-5) or peptides (10ug/ml) at 37⁰ C in
5% CO₂. Autologous B-LCLs were incubated overnight with optimal peptides (10
ug/ml), LFn-HIV (10 ug/ml) or recombinant vaccinia viruses (MOI 3- 5), washed
twice and added to effector cells at E:T ratio of 10:1 in the presence of co-stimulatory
10 anti-CD28 and anti-CD49d (1 ug/ml, Becton-Dickinson, San Jose, CA. Brefeldin A
(10 ul of 1mg/ml) was subsequently added and the cell culture was incubated at 37⁰C
for 6 hours and stained with saturating solution of isotype controls (IgG1 for APC,
PE, PerCP and IgG2b for FITC) or allophycocyanin (APC) –labeled CD3 monoclonal
antibody (Mab), phycoerythrin (PE) -labeled anti-CD8 Mab (Becton Dickinson).
15 After incubating in dark at 4⁰C for 20 minutes, the cells were washed twice with
FACS wash. 100ul of reagent A (Fix and Perm kit, Caltag Laboratories, Austria) was
added and after incubating in dark at room temperature (RT) for 20 minutes, the cells
were washed twice with FACS wash. 100 ul of reagent B (Fix and Perm kit) was then
added and cells were incubated at RT for 5 minutes. After adding FITC-IFN gamma
20 and peridinin chlorophyll protein (PerCP)-conjugated CD69 Mab (CD69 perCP)
antibody and incubating in dark at 4⁰C, the cells were washed twice with FACS wash
and analyzed on Becton Dickinson FACScalibur flow cytometer using the Cell Quest
software.

Samples were analyzed with four colors staining using the
25 FACScalibur Flow Cytometer (Becton Dickinson) and Cellquest software (Becton
Dickinson). Negative and positive controls used were unstimulated cells as well as
cells stimulated, respectively, with mitogen phematohemagglutinin PHA (0.25 ug/ml,
Murex Biotech).

5 Cell Lines and Culture Conditions.

Autologous Epstein-Barr virus (EBV)-transformed B lymphoblastoid as well as T1 and T2 (HLA-B60) cell lines were used as antigen presenting cells (APCs). Target cells were pulsed with the appropriate index peptides or LFn-HIV constructs or infected overnight with recombinant vaccinia viruses. AC13 (HLA B14
10 restricted p24 [DRFYKTLRA]) and KM (HLV B60-restricted Nef [KEKGGLEGL]) are Gag and Nef-specific clones (respectively) and AC2 (HLA B44 restricted gp120 [AENLWVTVY]). All were obtained from HIV infected individuals whose CTL responses are well characterized (Rosenberg, personal communication) and the Env-specific clone (SP 511) was generated from a seropositive Ugandan (16). PMBCs
15 used in the Elispot study were HIV-1 infected individuals from Senegal and Uganda (15,16). To investigate the effect of brefeldin A, cytochalasin B or chloroquine on MHC class I restricted presentation of LFn-HIV, APCs were labeled with the protein constructs, cultured in RPMI 1640m 10%FCS and cocultured with respective reagents for one hour, then washed and tested in the standard chromium release assay.

20 Elispot

Elispot assay was performed on cryopreserved PBMCs. 96-well nitrocellulose plates (Millititer, Millipore Corp., Bedford, MA) were pre-coated overnight in 4⁰C with 0.5 mg/ml of monoclonal antibody 1-D1K (Mabtech, Stockholm, Sweden). The plates were then washed 6x with phosphate-buffered saline
25 (PBS) and PBMCs were added at 50,000 cells/well and 25,000 cells/well in duplicate wells, respectively. The plates were incubated overnight at 37⁰C in 5% CO₂ and biotinylated monoclonal antibody anti-IFN-Mab (Mabtech) was then added at 0.5mg/ml for 100 minutes followed by streptavidin-ALP (Mabtech) for 1 hour at

5 room temperature. The plates were washed three times with PBS and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue (Sigma) added to develop the reaction. Tap water was added to stop the reaction after 15 minutes. Individual cytokine-producing cells were detected as dark spots, which were visualized and quantitated as SFC/well (spot forming colony/well). CTL frequency (CTLp) was calculated from the number
10 of spots subtracted from the control wells and averaged from the four wells. The final CTLp was reported as the average frequency per 10^6 cells. Responses were considered positive if the SFCs were at least twice that of control. Background SFC was on average less than 15/well.

Chromium release assay:

15 CD8+ CTL clones were stimulated with anti CD3 monoclonal antibody (12F6) in the presence of recombinant IL-2 and tested for activity within seven days. Target cells included autologous B-LCL or HLA-matched APCs infected with recombinant vaccinia virus (multiplicity of infection 3 to 5) expressing HIV-1 gene products or with LFn proteins (5 to 30 ug/ml) overnight and labeled with
20 radioactive chromium (^{51}Cr). Effector:target cell ratio (E:T) was 10:1 in a final volume of 200 ul with all assays performed in duplicate wells. Supernatant fluid was harvested after 4 hours and the percent specific lysis was determined from the formula: $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$. HIV-specific CTL activity was defined as 10% above
25 background/control. Spontaneous release was <30% of maximal release for all assays.

LFn-HIV mediates HIV-1 antigen entry and processing without PA:

5 To assess the ability of LFn-HIV to associate with MHC-I molecule on
cell surface, lymphoblastoid B cell lines (B-LCL) were sensitized with LFn-HIV in
the presence or absence of PA and tested in a standard chromium release assay using
several well characterized HIV-specific CTL clones (Figure 3). We found that CTL
recognized target cells sensitized with LFn-HIV comparably in the presence or
10 absence of PA. Lysis level was dose-dependent and achieved to levels comparable to
positive controls where B-LCLs were infected with recombinant vaccinia viruses
expressing the same HIV antigens. HIV-specific CTL activity was evident only when
targets had a longer incubation period with LFn-HIV (8 hours vs 1 hour) (data not
shown), suggesting a delay in the appearance of surface antigen and the requirement
15 for intracellular processing.

LFn-HIV is presented in the classical MHC-I pathway

We next verified that LFn-HIV was presented by the classical MHC-I
pathway, by demonstrating that the recognition of these constructs by CTLs was
HLA-I restricted (Figure 4). Two Nef- (KM) and Gag-specific (AC13) clones that are
20 HLA B60- and B14-restricted, respectively, were tested with HLA matched and
mismatched targets presenting LFn-Nef or LFn-p24. Only HLA-matched targets
demonstrated significant lysis, confirming the MHC-I presentation of LFn-HIV.

We further examined the potential mechanism of uptake and
processing of LFn-HIV. To determine whether the internalization of LFn-HIV at the
25 surface was actively processed, we pre-incubated B-LCL with 100 uM of cytochalasin
B (17,18), a phagocytosis inhibitor before adding LFn-HIV antigens. CTL recognition
was abrogated in the presence of cytochalasin B, suggesting an endocytosis-mediated
internalization process.

5 If exogenous LFn fusion protein was introduced and subsequently
processed in the cytosol, TAP proteins (Transporter associated with antigen
processing) would be required for the transport of peptides into the ER lumen for
binding to MHC-I (19-21). To evaluate the requirement for antigenic processing after
endocytosis, we tested the ability of LFn-HIV fusion proteins to sensitize the B60-
10 expressing cell lines T1 and T2 to lysis by CTL. T2 cell (22-24) is a derivative of T1
cells lacking the function of TAP, which transports peptides from the proteasome to
the ER lumen for binding to MHC-I (19,20). Recognition was abrogated in T2 cells
(Figure 4B), indicating the need for epitope transport after processing. Recognition of
this TAP deficient target by the same clone was maintained if the cells were pulsed
15 with the optimal epitope peptide directly to the surface “empty” MHC-I molecule,
bypassing the requirement for intracellular processing.

To further verify the mechanism of exogenous protein entry into the
MHC class I pathway, target cells were treated with brefeldin A, which inhibits
exocytosis of proteins from the ER and Golgi complexes and prevents newly
20 assembled peptide-MHC complexes from reaching the cell surface (25). The addition
of brefeldin A before presenting the target cells with LFn-HIV effectively blocks
recognition by CTLs (Figure 5). This finding, together with the earlier demonstration
for HLA restriction and TAP requirement, confirms that LFn-HIV was processed and
presented by B-LCL in a MHC class I pathway.

25 Processing of LFn-HIV is chloroquine insensitive

The presentation of many exogenous antigens on MHC-I molecule
involves proteolysis in the endocytic compartment (26-28) and the peptides
subsequently gain access to the cytosol where it enters the MHC class I pathway. To

5 investigate whether LFn mediated antigen presentation requires proteolysis in acidic vesicles, we treated the B-LCL with chloroquine during exposure to LFn-HIV (Figure 5). It is known that chloroquine raises the pH in the endosomal and lysosomal compartments thus inhibiting protein hydrolysis by cathepsins, which require an acidic environment for activity (29,30). Our finding suggested that phagosomes with
10 leaky properties are not requisite for the entry of LFn-HIV into the cytosol.

The addition of either cytochalasin B, brefeldin A or chloroquine did not affect recognition and lysis of target cells if targets were pulsed with the optimal 8-mer epitopes at the surface, demonstrating that these reagents did not affect CTL function nor target viability (Figure 5).

15 Comparing LFn-HIV with recombinant vaccinia virus in different CMI assays

Having demonstrated the ability of LFn-HIV to present CTL epitopes with MHC-I molecules at the cell surface, we proceeded to test the applicability of the LFn fusion proteins in various CMI assays, including the enzyme linked spot (Elispot) assay and flow-based intracellular interferon assay. These assays provide simpler and
20 more rapid estimate of T cell responses and theoretically better suited for large clinical testing, especially in context of vaccine studies. The main advantage of the Elispot assay is that the HIV-specific CTL response can be evaluated effectively and efficiently in large number of people (31). Currently various antigen stimuli are used in this assay, including recombinant vaccinia viruses expressing HIV antigens or
25 overlapping synthetic peptides. Cryopreserved PBMCs as well as CD8+ CTL clones from several HIV-1 positive individuals were tested in the Elispot assay, using LFn-p24, LFn-Nef or LFn-gp120 in parallel with recombinant vaccinia viruses expressing the same antigens. Comparable spot forming colonies were observed with LFn-HIV

5 and in some instances, less background spots was evident when compared to that with recombinant vaccinia virus (Figure 6).

Flow cytometry-based assay detects the intracellular accumulation of cytokines after in vitro stimulation of antigen-specific T cells. CTLs are stimulated with antigens and incubated with brefeldin A, which inhibits protein transport and
10 allow intracellular accumulation of newly synthesized cytokines such as IFN- γ . Surface markers (CD8, CD3) and intracellular staining of IFN- γ and CD69 allows detection of and quantification of specific T cell population against antigenic epitopes in the context of uncharacterized HLA-I backgrounds. A Nef-specific clone (KM) was stimulated with LFn-Nef and subsequently evaluated for intracellular production of
15 the interferon by flow analysis (Figure 7A). Nef-specific response was detected with LFn-Nef as well as recombinant vaccinia virus expressing Nef in a cell population that is CD8⁺. Freshly isolated, unstimulated PBMCs from an individual (AC2) with known gp120 epitope was sensitized with LFn-Env as well as with recombinant vaccinia virus expressing Env (rVV-Env). The percentage of intracellular IFN- γ in
20 the CD8⁺ cell population was higher in cells exposed to LFn-Env compared to those incubated with rVV-Env (Figure 7B).

EXAMPLE 2

LFn fusion proteins in the absence of PA are also capable of
25 sensitizing CTL target cells in a MHC-I restricted manner. This cytosolic delivery of LFn fusion proteins relies on functional transportation associated with intracellular antigen processing inside an antigen-presenting cell (Cao H, Agrawal D, Kushner N,

5 Touzjian N, Essex M, and Lu Y. *J. Infect. Dis.* 185:244-251 (2002)). We have now demonstrated that Green Florescence Protein (GFP) fused with LFn can enter into cells in the absence of PA. We further show that this PA-independent LFn delivery of exogenous GFP appears to be associated with cellular proteosome, which is consistent with the previous observation.

10 Construction and purification of LFn-GFP and GFP

 LFn-GFP fusion protein was constructed by insertion of the GFP open reading frame from pEGFP-C1 (Clontech) into the LFn expression vector described in previous studies (Lu, Y., R. et al., *Proc. Natl. Acad. Sci. USA* 97:8027-32 (2000)). The fusion protein is soluble in bacterial cell extract and can be purified in one step
15 affinity chromatography. The purified fusion protein has a molecular weight of approximately 55 kD and its solution has a bright green color (data not shown). In order to have an appropriate control for the experiments, GFP alone was constructed into the same bacterial expression vector, pET15b, so that the only difference in the expression, purification, and use of GFP and LFn-GFP is the lack of the LFn
20 sequences in GFP.

LFn-GFP enters CHO cells whereas GFP cannot

 CHO cells were cultured in collagen treated chamber slides to reach 80% confluence and were then incubated with purified LFn-GFP for one or two hours, followed by washing extensively with PBS and PBS plus proteases to remove
25 membrane bound proteins. The cells were then stained with the anti-transferrin antibodies and fixed with paraformaldehyde. The slides were examined by confocal microscopy for green color (GFP) and red color (anti-transferrin), respectively. A third image is presented for each field by super-expose the green image with the red

5 image of the same cells. Consequently, a yellow spot indicates that the GFP may be in the same spot with the transferrin protein, thus providing a reference to the location of GFP inside the cells. As shown in Figure 10, a significant number of green spots were visible in cells treated with LFn-GFP for one hour, suggesting the LFn-GFP can indeed enter into cells. Under the exactly same experimental conditions, GFP-treated
10 cells showed few green spots (Figure 11).

Location of LFn-GFP inside treated-cells

Using the identical experimental procedures described above for Figures 10 – 11, we substituted the anti-transferrin antibodies with an anti-lysosome antibody (Figure 12), an anti-endosome antibody (Figure 13), an anti-Golgi antibody
15 (Figure 14), and an anti-proteosome antibody (Figure 15), respectively. By comparing these different images, the super-exposed images that have the most yellow spots are those shown in Figure 15 in which the green spots representing the intracellular LFn-GFP overlap significantly with the red spots representing cellular proteosome.

20 The presence of PA does not enhance the cellular intake of LFn-GFP

We further examined if the presence of PA would enhance the intake of LFn-GFP under the experimental conditions used for Figures 10 – 15. As shown in Figure 16, adding PA did not increase the number of green spots inside cells in comparison with those in the absence of PA. Moreover, it seems that the presence of
25 PA might have reduced the amount of yellow spots. Apparently, it is likely that under these conditions these yellow spots still represent the PA-independent entry of LFn-GFP.

5 EXAMPLE 3

Deletion of the N-terminal half of LFn, and the adjuvant effect of Alum on LFn
immunization

Additional animal immunization data demonstrates that the N-terminal half of LFn, which contains the PA binding domain, can be deleted with no negative
10 effect on the antigen delivery. Quite unexpectedly, we found that the addition of Alum could significantly enhance the CTL induction by the LFn fusion proteins.

Despite many unsuccessful attempts in the past two years, we have been unable to show that LFn-p24 in the presence of PA could stimulate CTL in immunized BALB/c mice. Previous studies have shown that LFn fusion proteins such
15 as LFn-V3, LFn-LLO, and LFn-OVA are capable of stimulating specific CTL in mice (Lu, Y., et al. *Proc. Natl. Acad. Sci. USA* 97:8027-32 (2000); Ballard, J. D. et al., *Proc. Natl. Acad. Sci. USA* 93, 12531-12534 (1996); Ballard, J. D., et al., *Infect. Immun.* 66, 615-619 (1998)). These fusion proteins carry only 12 to 33 amino acids, whereas LFn-p24 carries about 230 amino acids. Thus the size of the inserted
20 antigens may make the difference. We decided to test if adding immune adjuvants could improve the immunogenicity of LFn-p24. Among the reagents we tested, which include recombinant IL2, Ig-IL2, CpG, Alum, and others, Alum unexpectedly showed the best adjuvant activity. It is widely believed that certain experimental adjuvants, such as QS21 and PCPP can enhance cell-mediated immune responses,
25 whereas Alum actually inhibits the CTL induction (Schiembeck, R., et al., *J. Immunol.* 152:1110-1119 (1994); Barouch, D.H. et al, *Science* 290:486 (2000); Davis, H.L. et al., *J. Immunol.* 160:870-876 (1998); Payne, L.G. et al., *Dev. Biol. Stand.* 92:79-87 (1998)).

5 In Figure 17, three groups of BALB/c mice, four in each group, were immunized i.p. with different antigen formulations, respectively (15 ug LFn-p24 plus 4 ug PA, 15 ug LFn-p24 plus 4 ug PA plus Alum, 15 LFn-p24 only plus Alum). At one-week post the immunization, splenic mononuclear cells from immunized BALB/c mice were used as the source of CTL. The CTL in the splenic cultures were activated
10 in vitro by culturing with gamma-irradiated and peptide-pulsed BALB/c splenocytes from un-immunized animals. After 6 days of culture in a 37°C CO₂ incubator, mature CTL (effector cells) were tested for their ability to lyse either ⁵¹Cr-labeled P20 peptide-pulsed P815 cells (positive targets) or ⁵¹Cr-labeled medium-pulsed cells (negative targets). The percent lysis shown were subtracted from the background
15 lysis of negative targets and presented as the average of each group.

As shown in Figure 17, with the addition of Alum, we were able to show that LFn-p24 can stimulate specific CTL in immunized mice in the presence or absence of PA. These results further demonstrate that the presence of PA is not required for the antigen delivery in vivo.

20 LFn fusion proteins where the N-terminal half of LFn has been deleted are still active

In an effort to confirm that LFn-p24 was able to elicit CTL in mice in the absence of PA, we constructed a mutated LFn fusion protein (MLFn-p24) in which the N-terminal half of LFn (1-149) was deleted to abolish its ability to bind PA. We conformed this by using an experimental method specially designed to test PA-
25 dependent membrane translocation. In brief, trypsin-nicked PA was incubated with CHO-K1 cells for 2 hours at 4° C. The cells were then washed with cold PBS and incubated with ³⁵S-labeled LFn-NG or LFn-ENV for 2 hours at 4° C. The cells were

5 washed extensively and exposed to MES/gluconate buffer (pH 4.8) at 37° C for 2 minutes. Pronase E or buffer was then added to digest surface bound LFn fusion proteins that had not been internalized. The cells were then washed again, lysed, and counted. The percentage of the protein translocated by PA was calculated according to the following formula. The ratio = 100x (counts in the presence of PA and the Pronase
10 treated cells - counts in the absence of PA and the Pronase treated cells)/(counts in the presence of PA and in mock treated cells - counts in the absence of PA and the mock treated cells). In this particular assay, PA translocated as high as 72% of the membrane bound LFn into the cells, whereas the amount of the MLFn translocated by PA was undetectable.

15 We then compared the CTL induction by LFn-p24 with MLFn-p24 in immunized mice. In Figure 18, three groups of BALB/c mice, four in each group, were immunized i.p. with 15 µg LFn-p24, 15 µg MLFn-p24, and 15 µg p24, respectively. The CTL in splenic tissues were tested one after the immunization.

As shown in Figure 18, both LFn-p24 and MLFn-p24 stimulated
20 significant CTL activity after only one immunization. In fact, we repeatedly observed an improved CTL induction by MLFn-p24 compared to that induced by LFn-p24. This experiment also demonstrates that the C-terminal half of LFn (150-253) is indeed responsible for the intracellular antigen delivery, as the deletion of the MLFn sequence from the antigen (p24) abolishes the efficient CTL induction.

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[001] All references described herein are incorporated herein by reference.

5 We claim:

1. A method of delivering a target antigen to the cytosol of a cell, comprising binding the target antigen to a transport factor, wherein the transport factor contains a fragment of a bipartite protein exotoxin that is not toxic to the cell, and wherein protective antigen (PA) is not used.
- 10 2. The method of claim 1, wherein the transport factor comprises SEQ ID NO:2 (LFn) or a portion thereof.
3. The method of claim 2, wherein the transport factor is a fragment that does not contain the portion of SEQ ID NO:2 that binds PA.
4. The method of claim 3, wherein the transport factor is the 80
15 carboxy-most amino acids of LFn.
5. The method of claim 3, wherein the transport factor does not contain amino acids 1-149 of SEQ ID NO:2.
6. The method of claim 3, wherein the transport factor is encoded by SEQ ID NO:3.
- 20 7. The method of claim 1, wherein the transport factor is a fragment of at least 80 amino acids that has at least 80% homology to SEQ ID NO:2.
8. The method of claim 1, wherein the transport factor a fragment is 350 amino acids or less.
9. The method of claim 1, wherein the transport factor is 300
25 amino acids or less.

5 10. The method of claim 1, wherein the transport factor is 250
amino acids or less.

 11. The method of claim 1, wherein the transport factor is 105
amino acids or less.

 12. The method of claim 11, wherein the transport factor is amino
10 acids 150-253 of SEQ ID NO:2 or less.

 13. The method of claim 1, wherein the target antigen is selected
from the group consisting of viral antigens, bacterial antigens, and tumor antigens.

 14. The method of claim 13, wherein the viral antigen is an HIV
antigen.

15 15. The method of claim 1, wherein the transport factor is bound to
the target antigen by expression of a fusion polypeptide wherein a single nucleic acid
coding sequence encodes both the transport factor and the target antigen.

 16. The method of claim 1, wherein the transport factor is bound to
the target antigen by a chemical linkage.

20 17. An isolated polypeptide comprising a target antigen and a
transport factor, wherein the transport factor contains a fragment of a bipartite protein
exotoxin that is not toxic to the cell and functions to deliver the target antigen to the
cytosol of a cell.

 18. The isolated polypeptide of claim 17, wherein the transport
25 factor does not contain a domain that binds to a corresponding protective antigen
(PA).

- 5 19. An isolated DNA encoding the polypeptide of claim 18.
20. The isolated DNA of claim 16 further comprising a 5'-flanking
region containing at least one promoter sequence for expression of the peptide.
21. A vector containing the isolated DNA of claim 20.
22. A pharmaceutical composition comprising an immunogenic
10 amount of an isolated polypeptide comprising a target antigen and a transport factor,
wherein the transport factor contains a fragment of a bipartite protein exotoxin that is
not toxic to the cell and functions to deliver the target antigen to the cytosol of a cell,
and wherein a corresponding protective antigen is not present.
23. The pharmaceutical composition of claim 22, further
15 comprising an adjuvant.
24. The pharmaceutical composition of claim 23, wherein the
adjuvant is Alum.
25. A method of generating a cell mediated immune response in a
mammal, comprising administering to the mammal the pharmaceutical composition of
20 claim 22.
26. The method of claim 25, wherein the pharmaceutical
composition is administered by subcutaneous or intramuscular administration.
27. The method of claim 25, wherein the pharmaceutical
composition is administered by oral ingestion.

5 28. A method of producing a protein, comprising expressing the isolated nucleic acid of claim 18 in a cell.

 29. The method of claim 28, wherein the cell is selected from the group consisting of bacterial cells, insect cells, and mammalian cells.

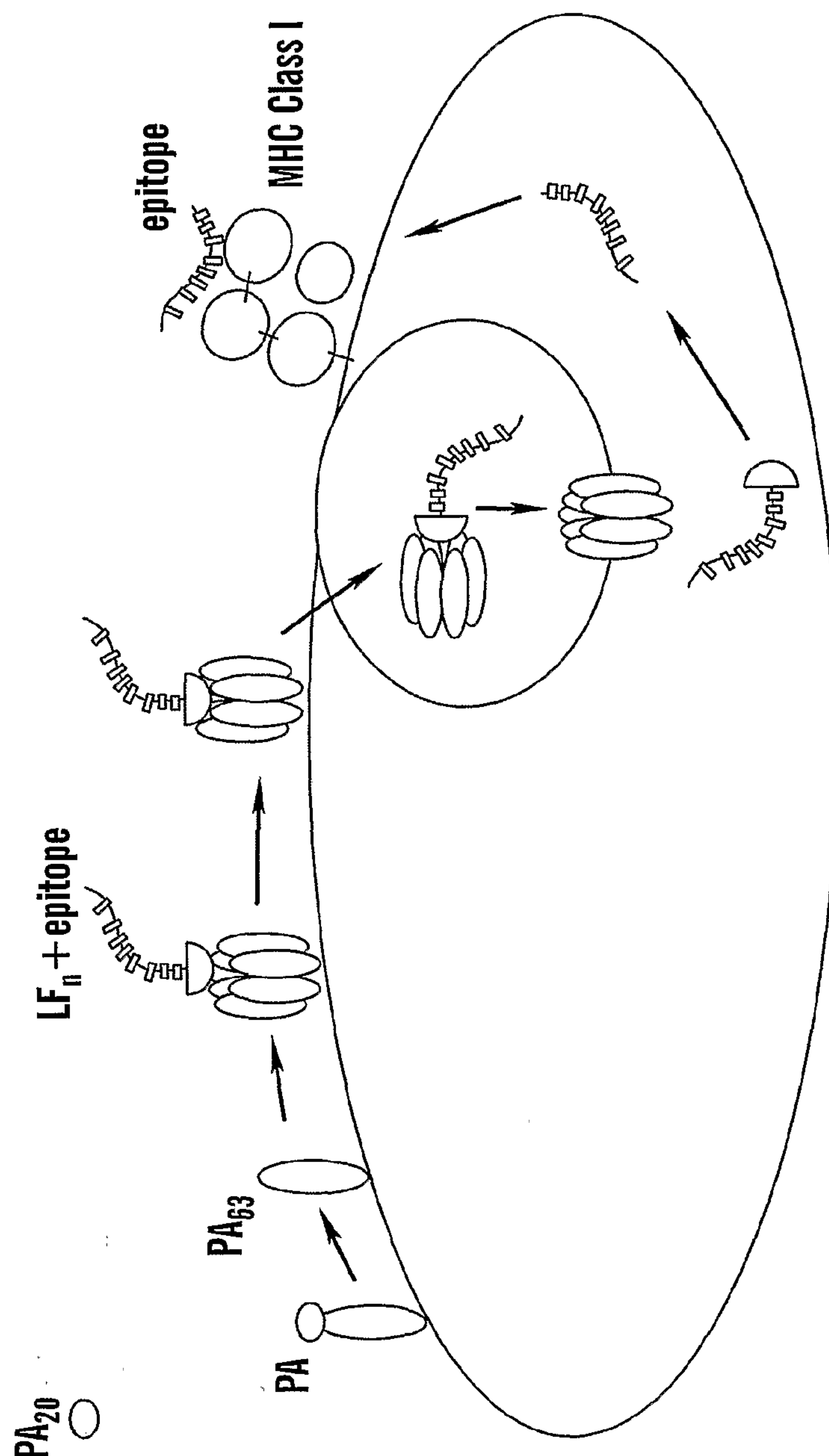
 30. The method of claim 29, wherein the cell is a bacterial cell.

10 31. A method for measuring a cell mediated immune response, comprising using the polypeptide of claim 17 in a cell mediated immune response assay.

 32. The method of claim 31, wherein the cell mediated immune response assay is selected from the group consisting of Elispot assays and flow-based
15 intracellular cytokine assays.

 33. A kit for measuring cell mediated immune responses in vitro, comprising the novel polypeptide of claim 17.

1/28

**FIG. 1**

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LF

1 mnikkefikv ismsclvtai tlgpvpfipl vqgagghgdv gmhvkekekn kdenkrkdee
 61 rnktqeehlk eimkhivkie vkgeeavkke aaekllekvp sdvlemykai ggkiyivdgd
 121 itkhisleal sedkkkikdi ygkdallheh yvyakegyep vlviqssedy ventekaln
 181 yyeigkilsr dilskinqpy qkfldvInti knasdsdggd llftnqlkeh ptdfsvefle
 241 qnsnevqevf akafayyiep qhrdvlqlya peafnymdkf neqeinlsle elkdqrmIsr
 301 yekwekikqh yqhwsdsIsle egrglkkIq ipiepkddi ihslsqeeke llkriqidss
 361 dflsteekef lkkIqidird slseeeekell nriqvdsnp lsekekefik kklIdiqpyd
 421 inqrlqdtgg lidspsinld vrkqyrIdiQ nidallhQsi gstlynkiyl yenmninnlt
 481 atlgadlvds tdntkinrgi fnefkknfky sissnymivd inerpaldne rlkwriqlsp
 541 dtragyleng klilqrnigl eikdvqiikq sekeyirida kvvpkskidt kiQeaqlnin
 601 qewnkalglp kytklitfnv hnryasnive sayliInewk nniqsdlIkk vtynIvdgng
 661 rfvftditlp niaeQythqd eiyeqvhskg lyvpesrsil lhgpskgvel rndsegfihe
 721 fghavddyag ylldknqsdI vtnsk kfidi fkeegsnIts ygrtneaeff aeafrlmhst
 781 dhaerlkvqk napktfqfin dqikfiins

FIG. 2A

LFn

1 mnikkefikv ismsclvtai tlgpvpfipl vqgagghgdv gmhvkekekn kdenkrkdee
 61 rnktqeehlk eimkhivkie vkgeeavkke aaekllekvp sdvlemykai ggkiyivdgd
 121 itkhisleal sedkkkikdi ygkdallheh yvyakegyep vlviqssedy ventekaln
 181 yyeigkilsr dilskinqpy qkfldvInti knasdsdggd llftnqlkeh ptdfsvefle
 241 qnsnevqevf akafayyiepqhrdvlqlya peafnymdkf neqeinlsI

FIG. 2B

Fragment 3

gkilsr dilskinqpy qkfldvInti knasdsdggd llftnqlkeh ptdfsvefle qnsnevqevf
 akafayyiep qhrdvlqlya peafnymdkf neqeinls

FIG. 2C

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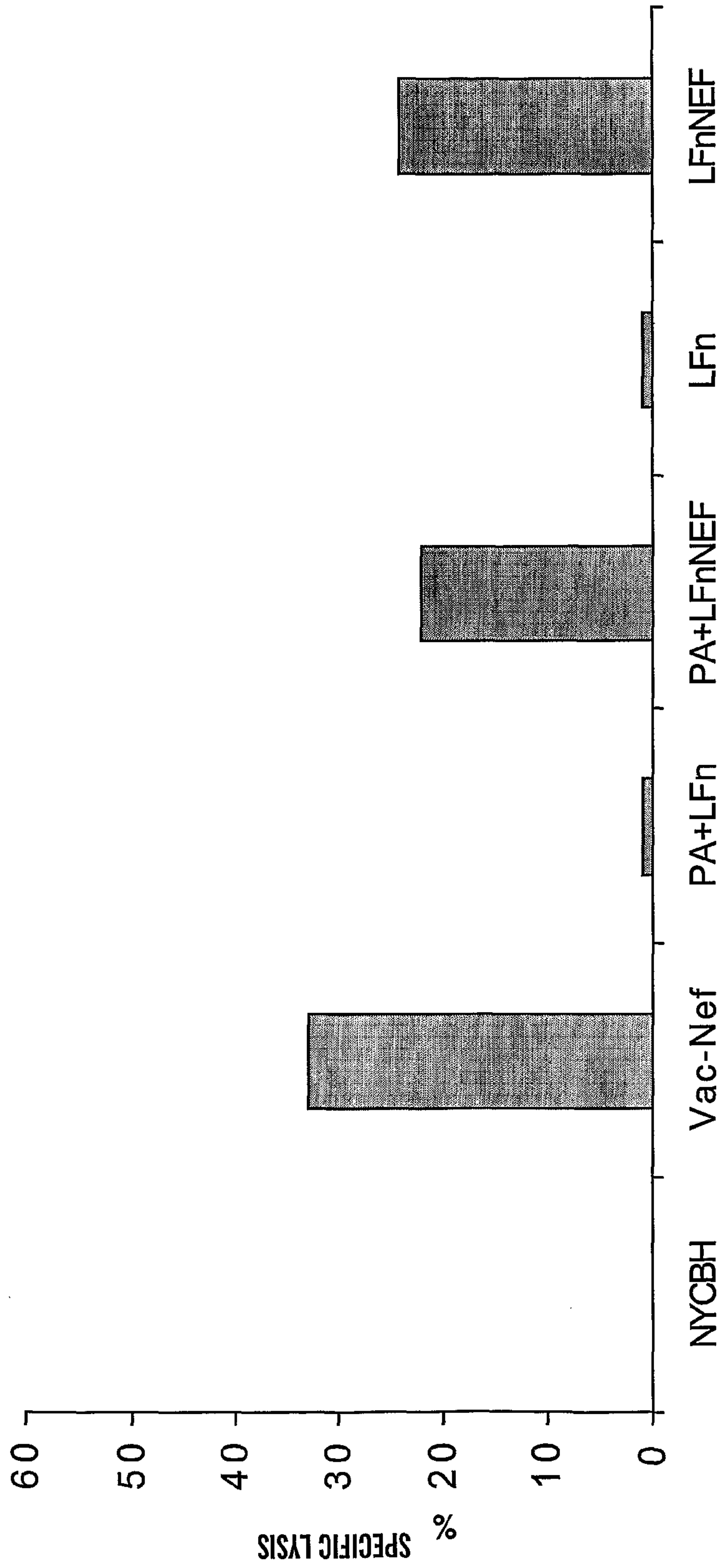


FIG. 3A

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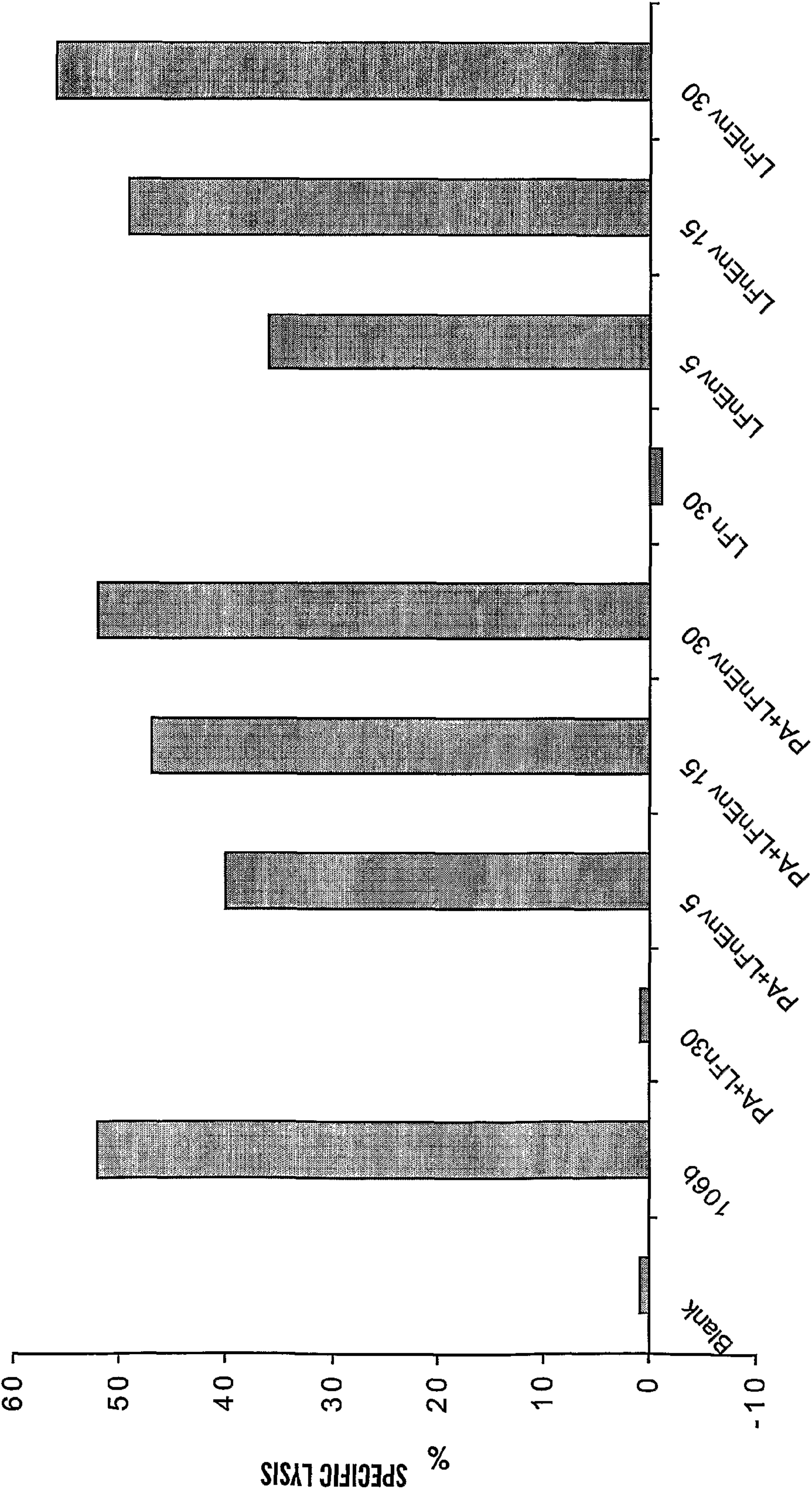
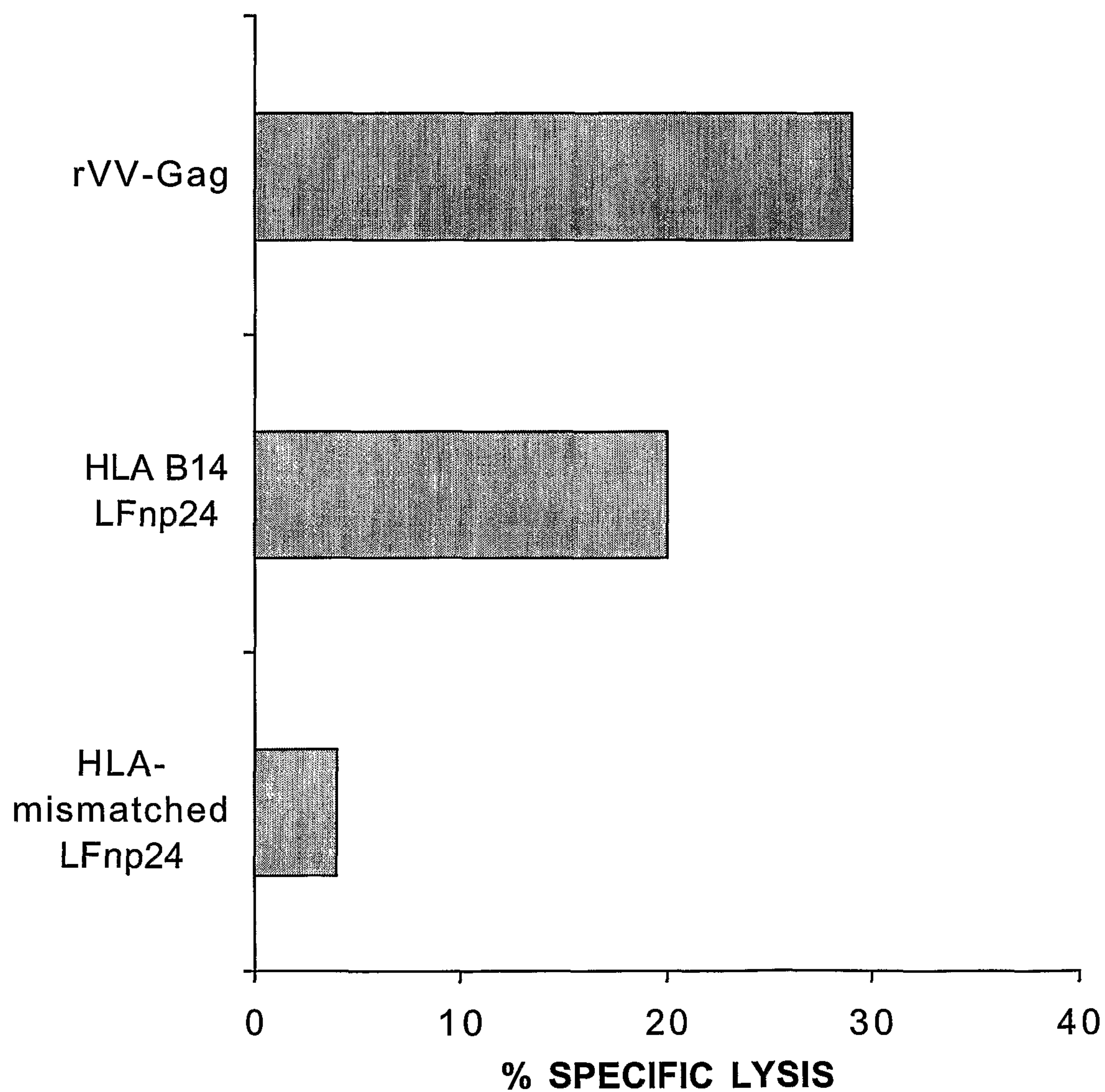


FIG. 3B

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B14 Gag CTL

**FIG. 4A**

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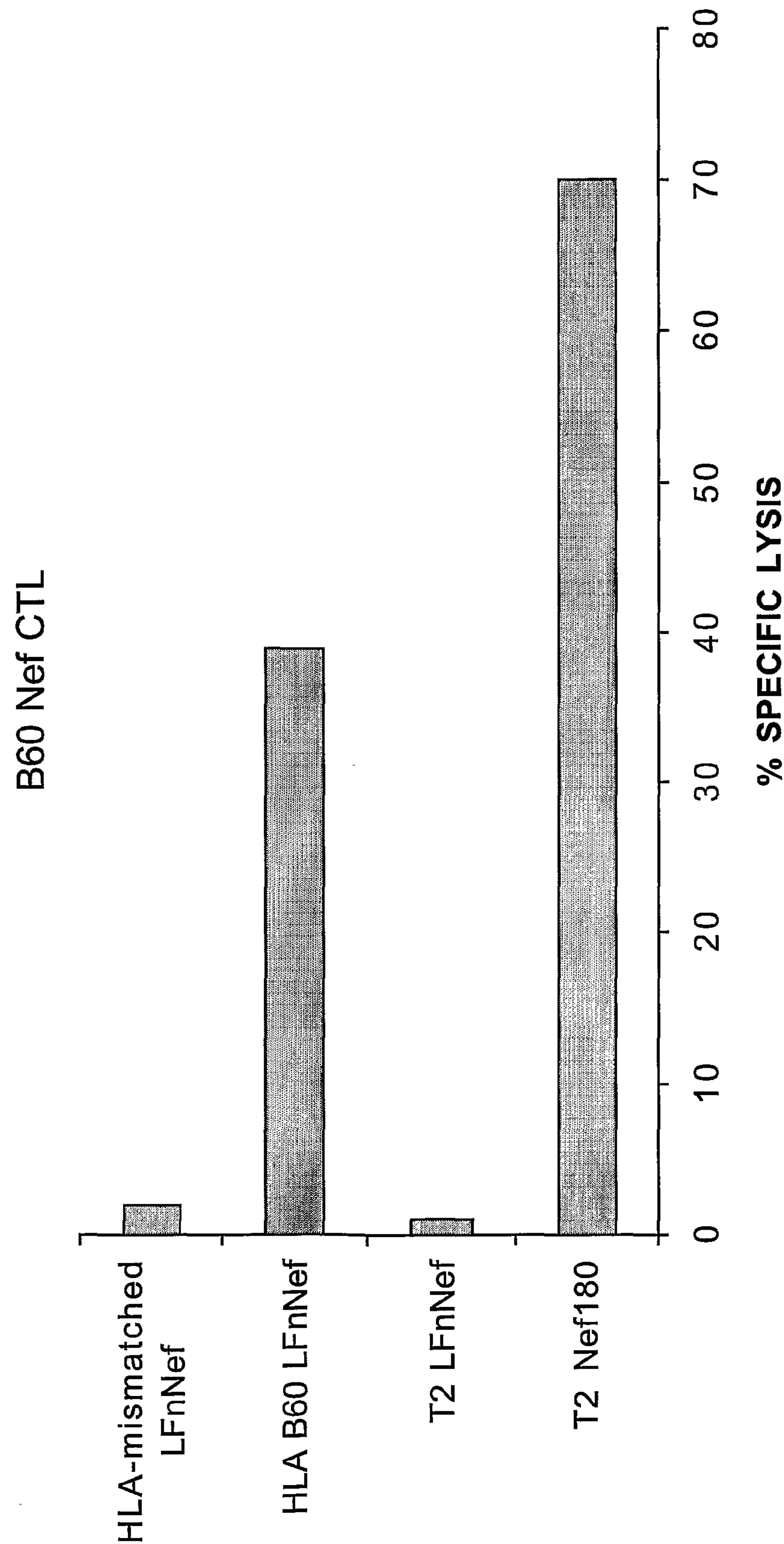


FIG. 4B

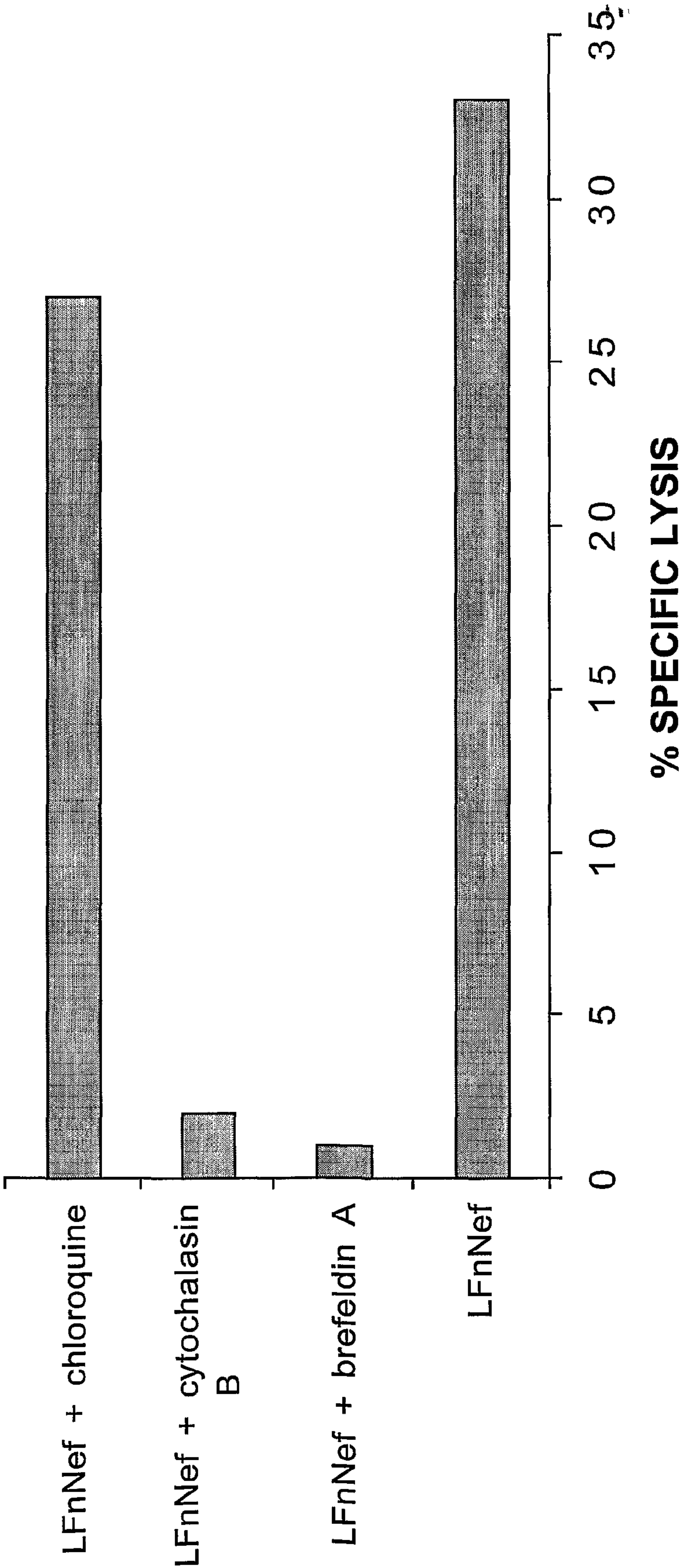


FIG. 5A

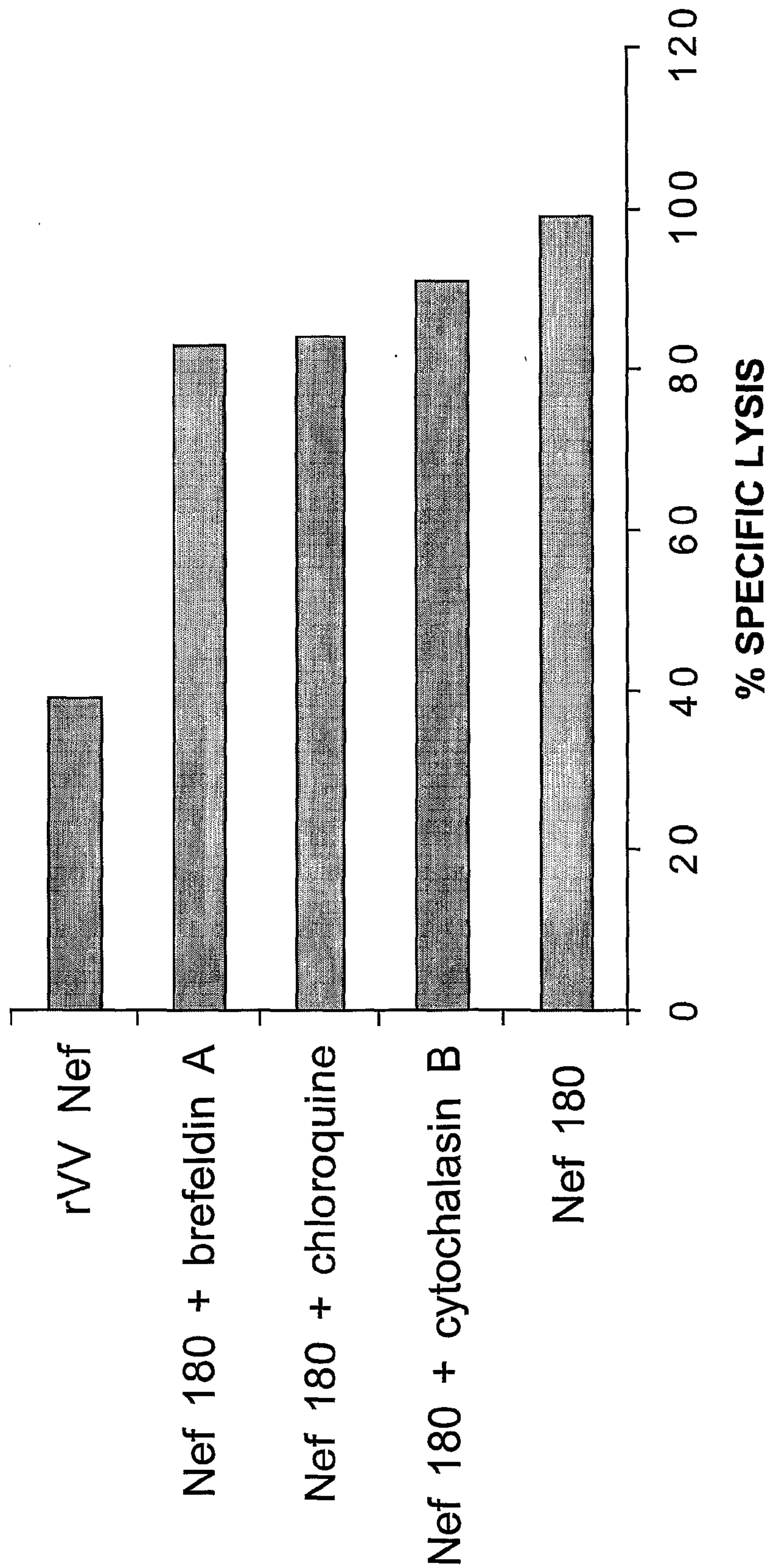
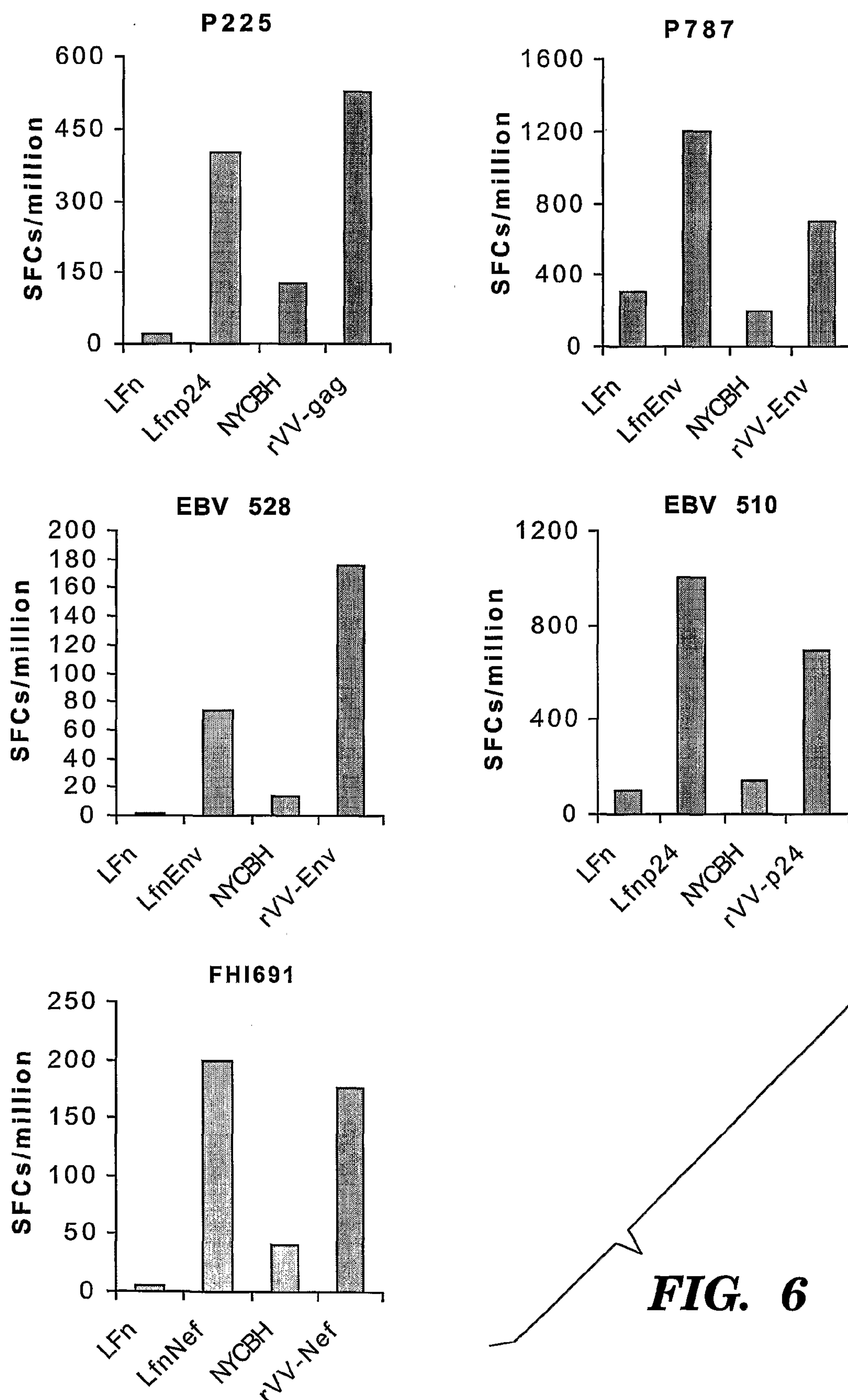
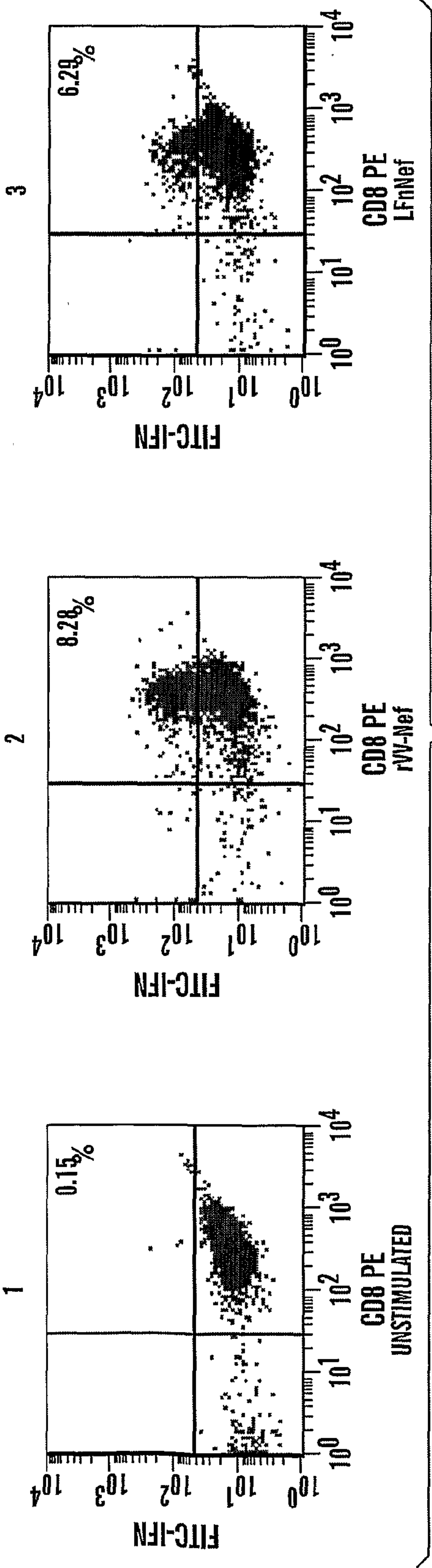


FIG. 5B

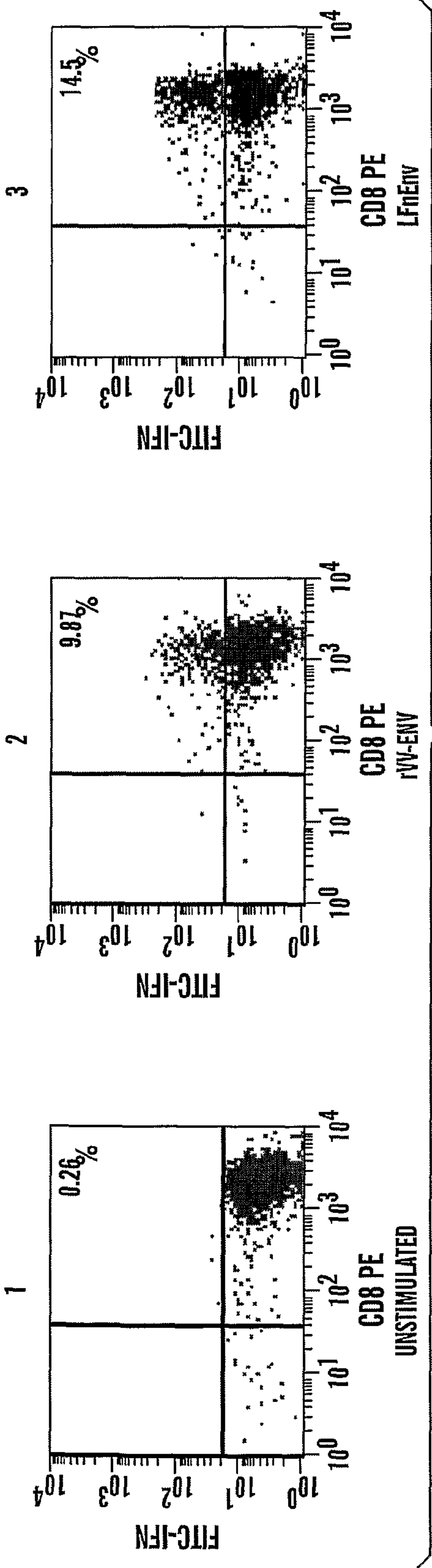
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**FIG. 6**

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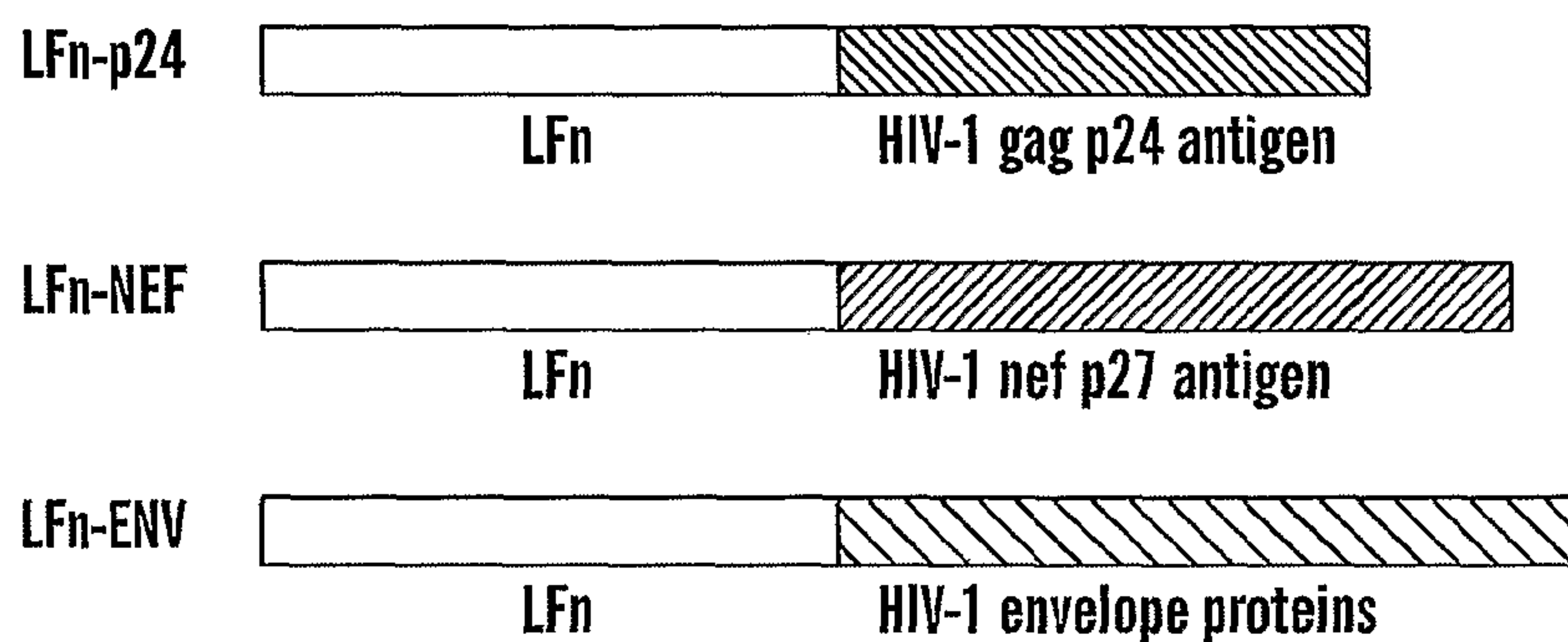
NEF-SPECIFIC CTL
FIG. 7A



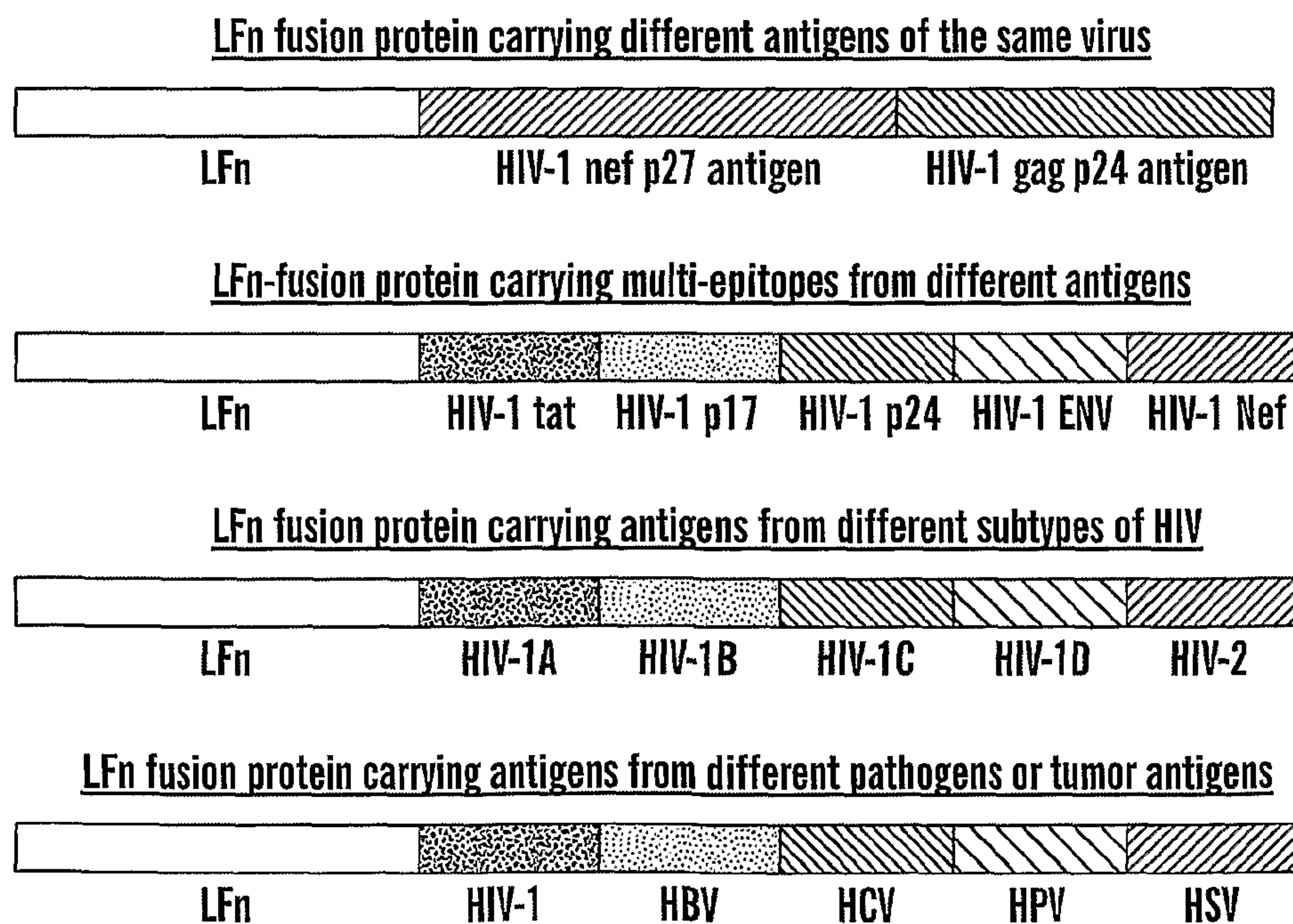
ENV-SPECIFIC CTL
FIG. 7B

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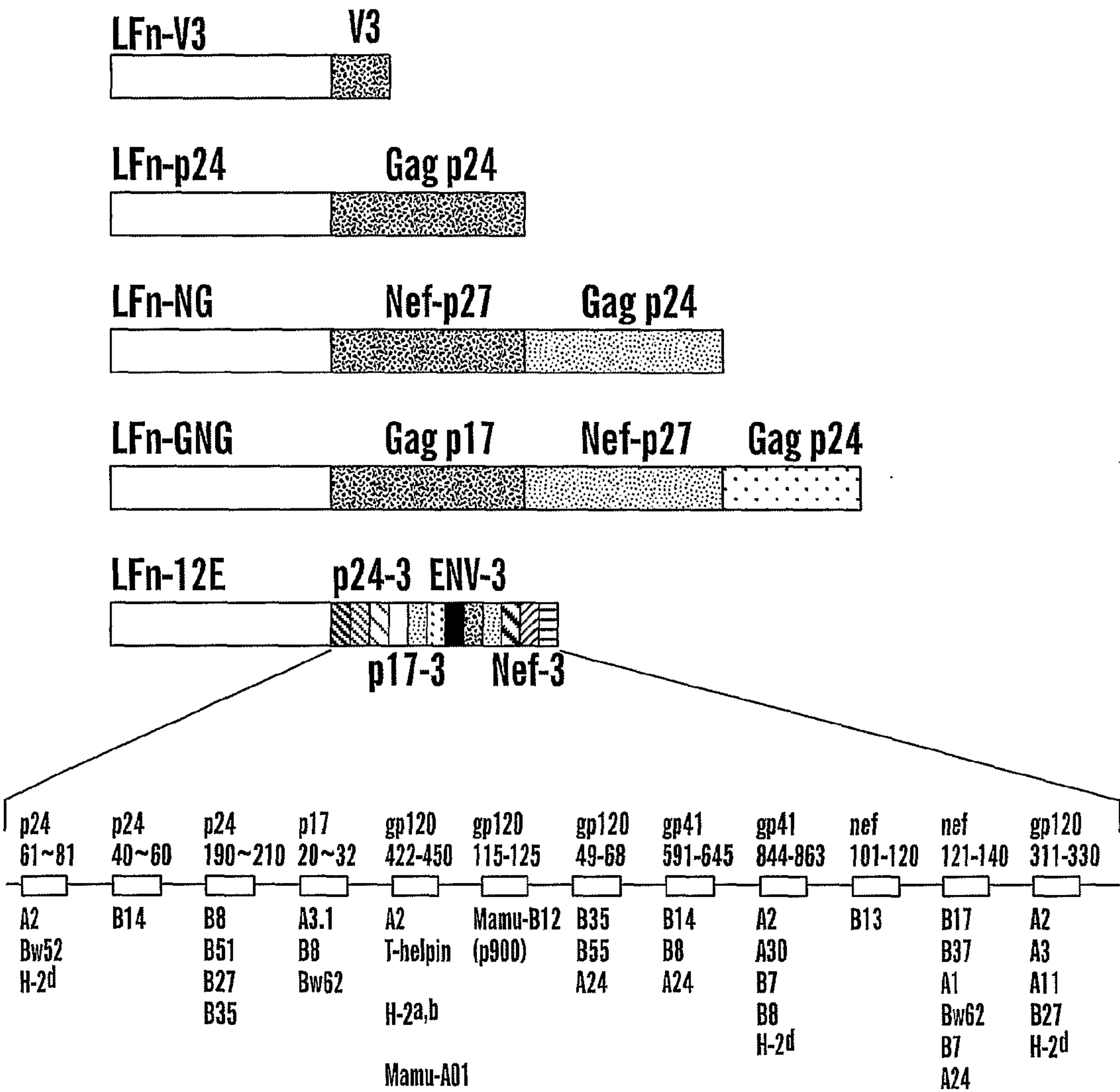
LFn fusion proteins used in this report

**FIG. 8A**

Proposed LFn-fusion protein for commercial applications

**FIG. 8B**

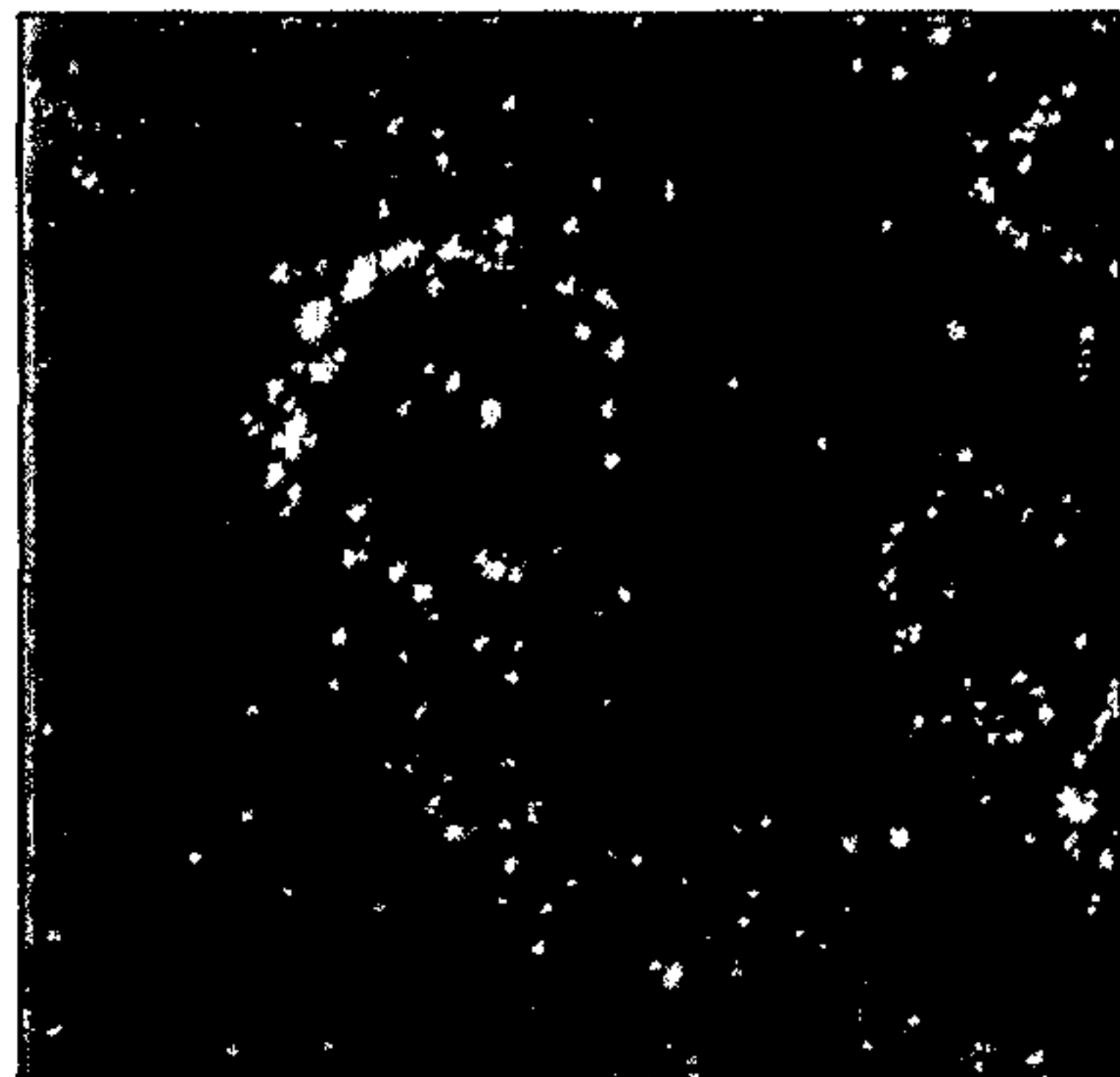
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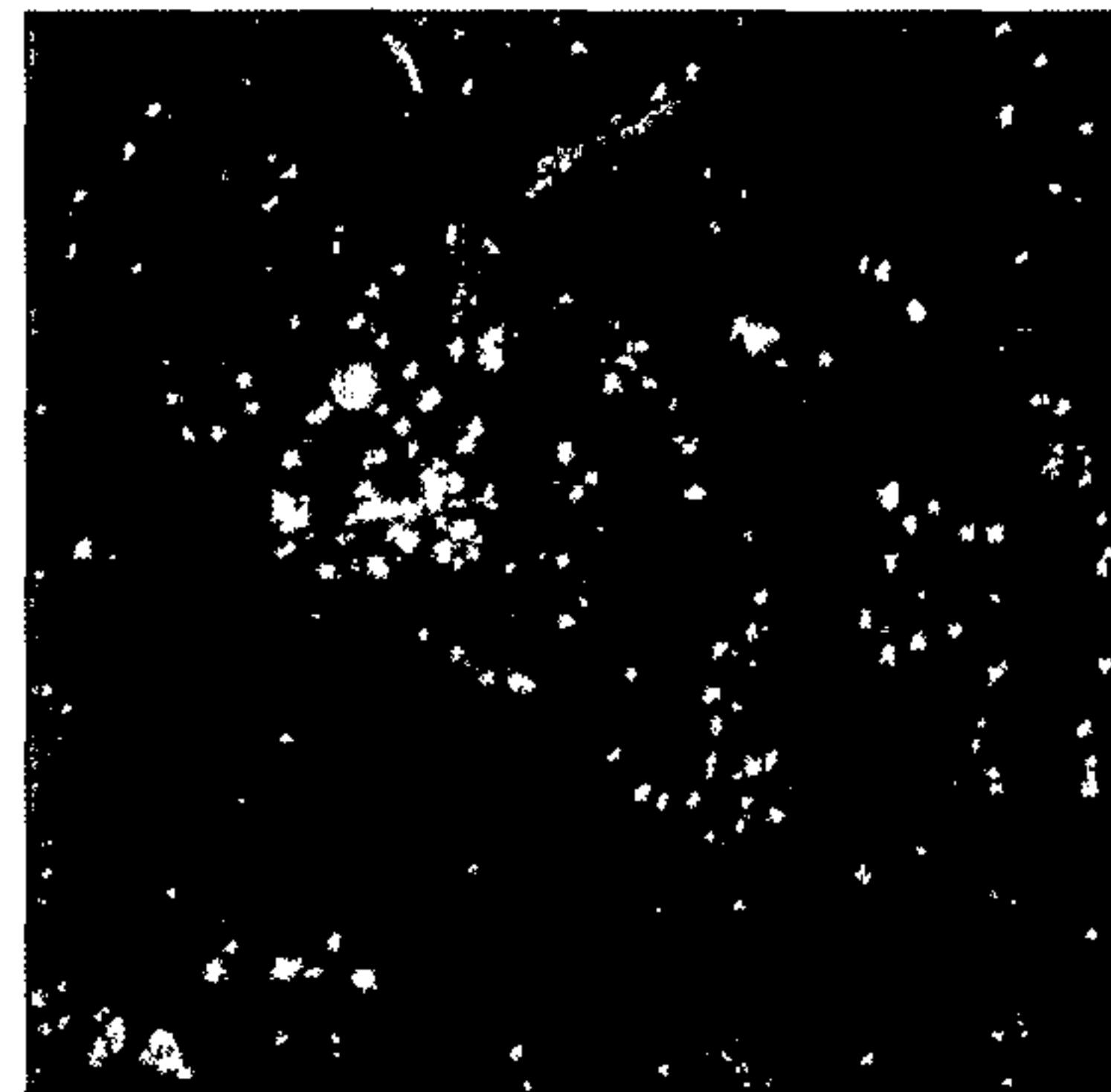
Ag	E#	HUMAN	MOUSE	MONKEY
p24	3	A2, B8, B14, B27, B35, B51, Bw52,	H-2d	0
p17	1	A3.1, B8, Bw62	0	0
gp120	4	A2, A24, A3, A11, B35, B55, B27,	H-2a,b, H-2d	Mamu-A01, Mamu-B12
gp41	2	A2, A24, A30, B7, B8, B14,	H-2d	0
nef	2	A1, A24, B7, B13, B17, B37, Bw62,	0	0

FIG. 9

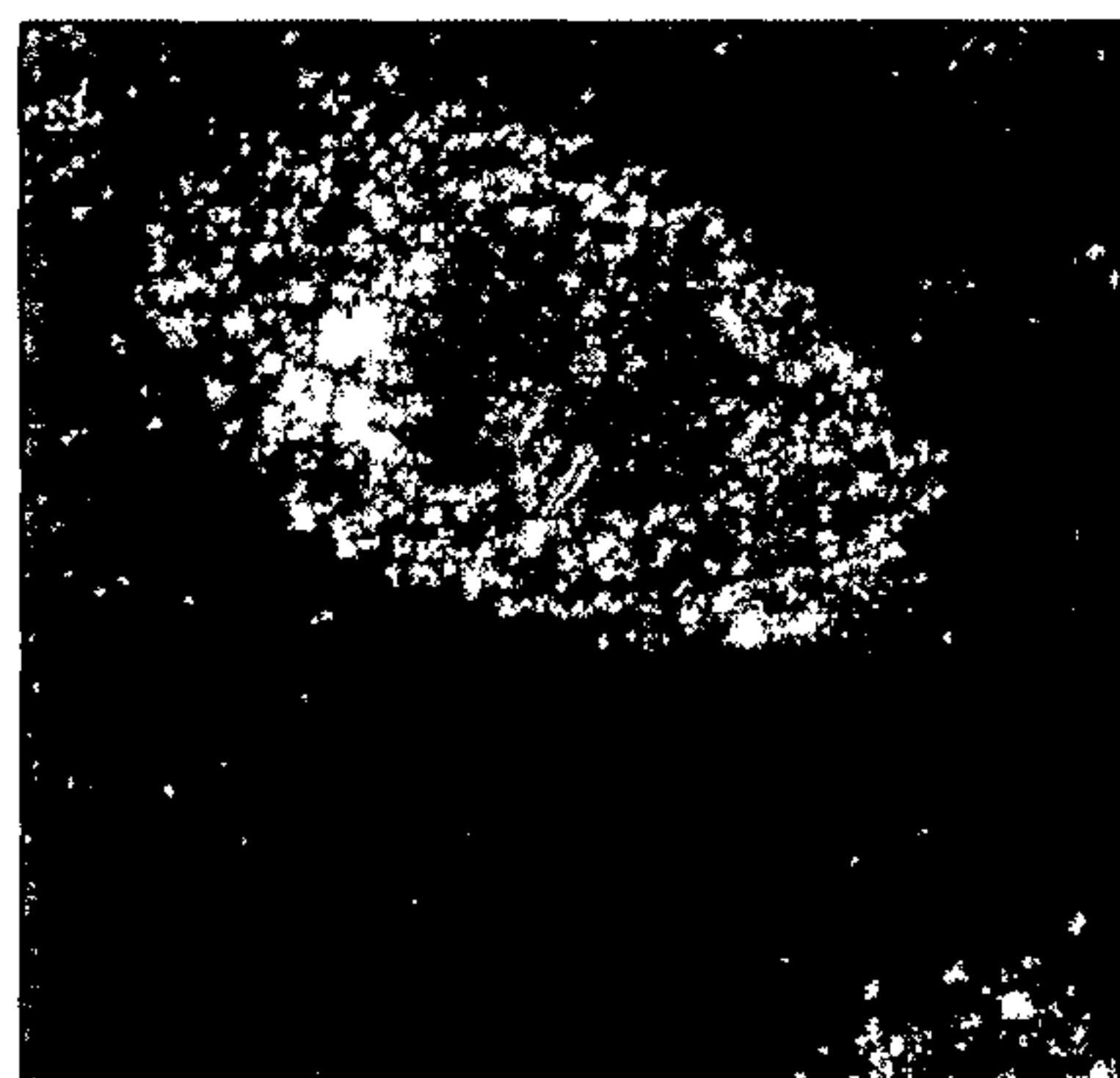
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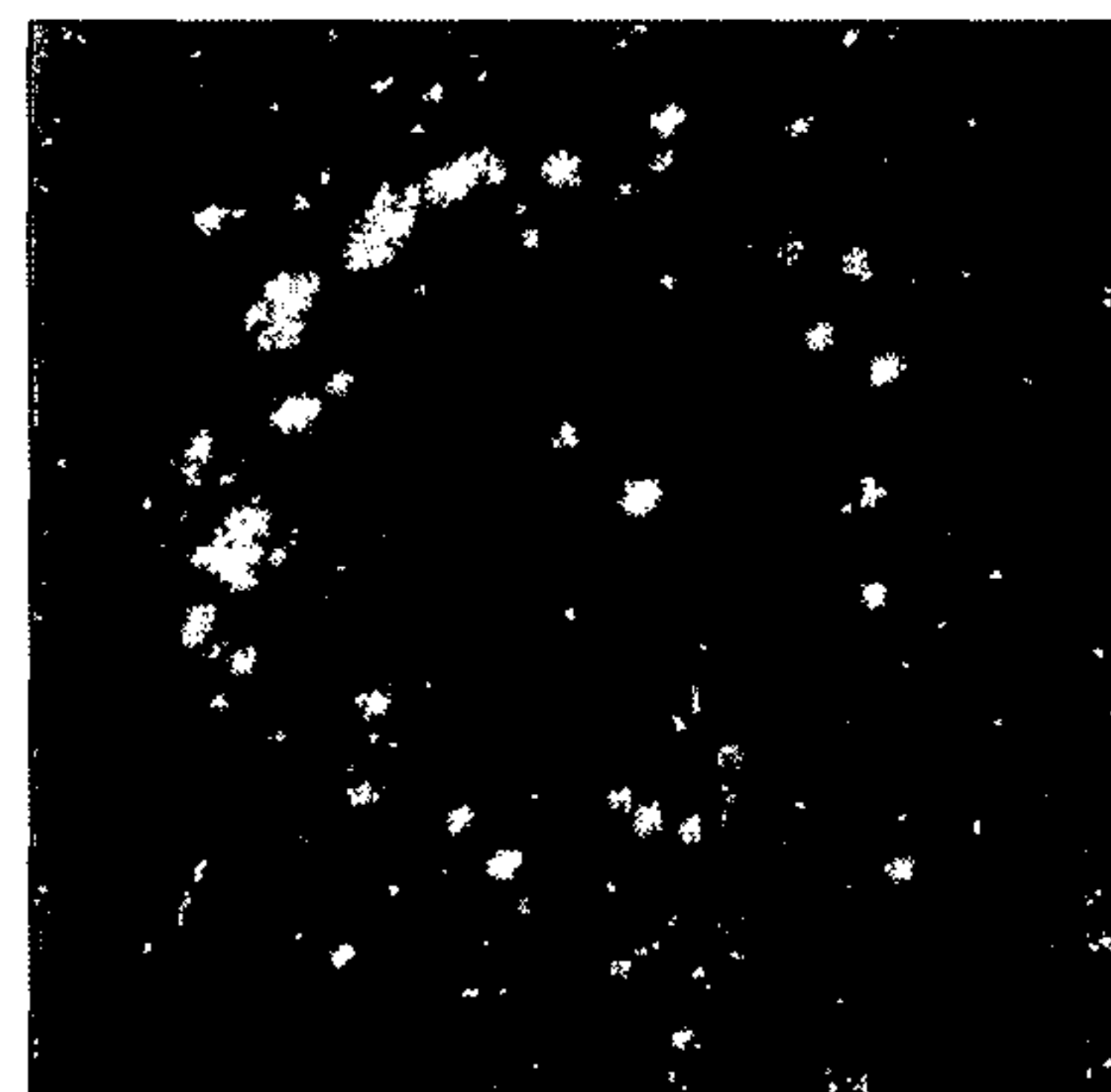
LN-GFP @37 C for 1 hr

FIG. 10A

LN-GFP @37 C for 1 hr

FIG. 10B

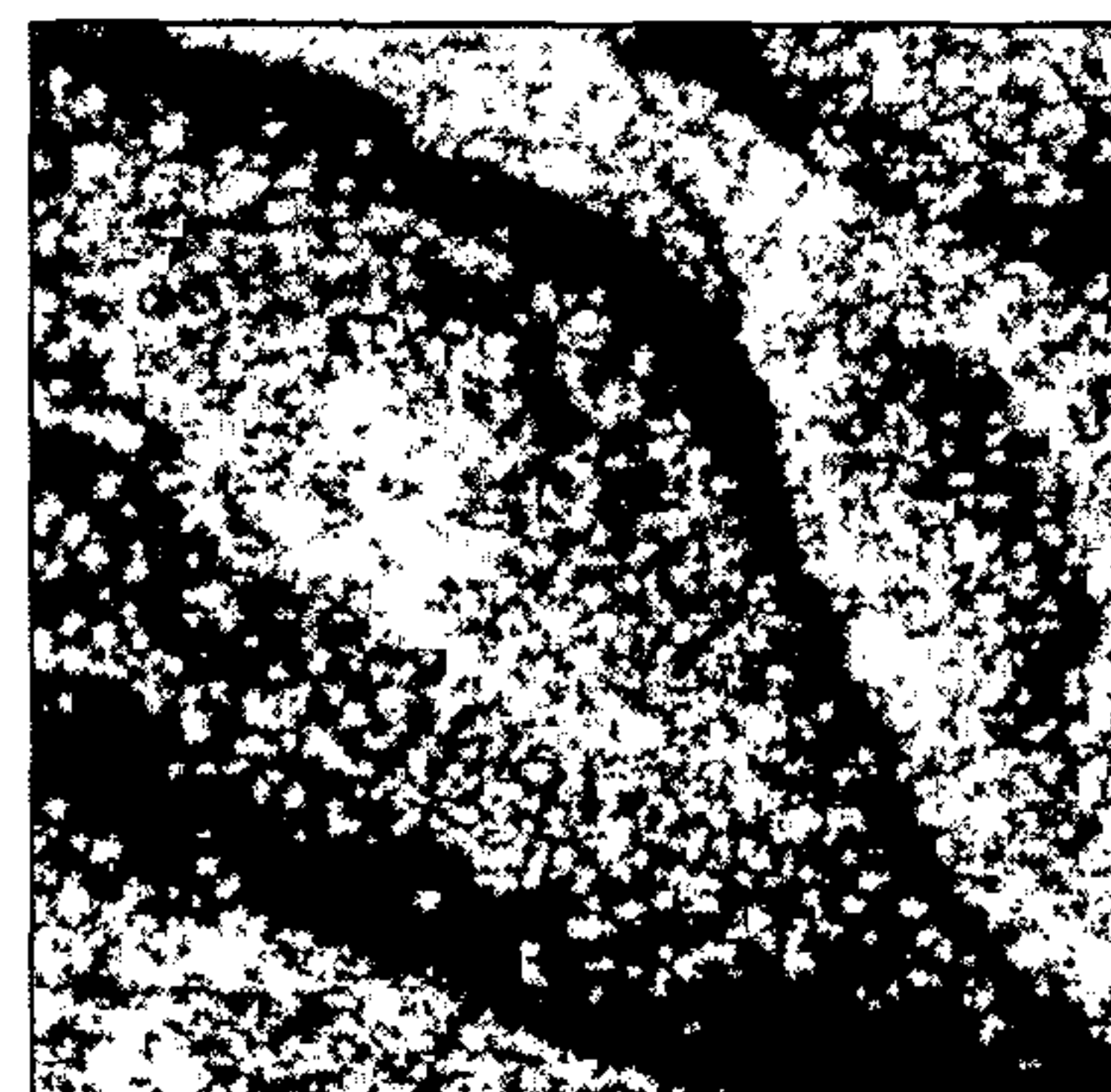
LN-GFP @37 C for 1 hr

FIG. 10C

LN-GFP @37 C for 1 hr

FIG. 10D

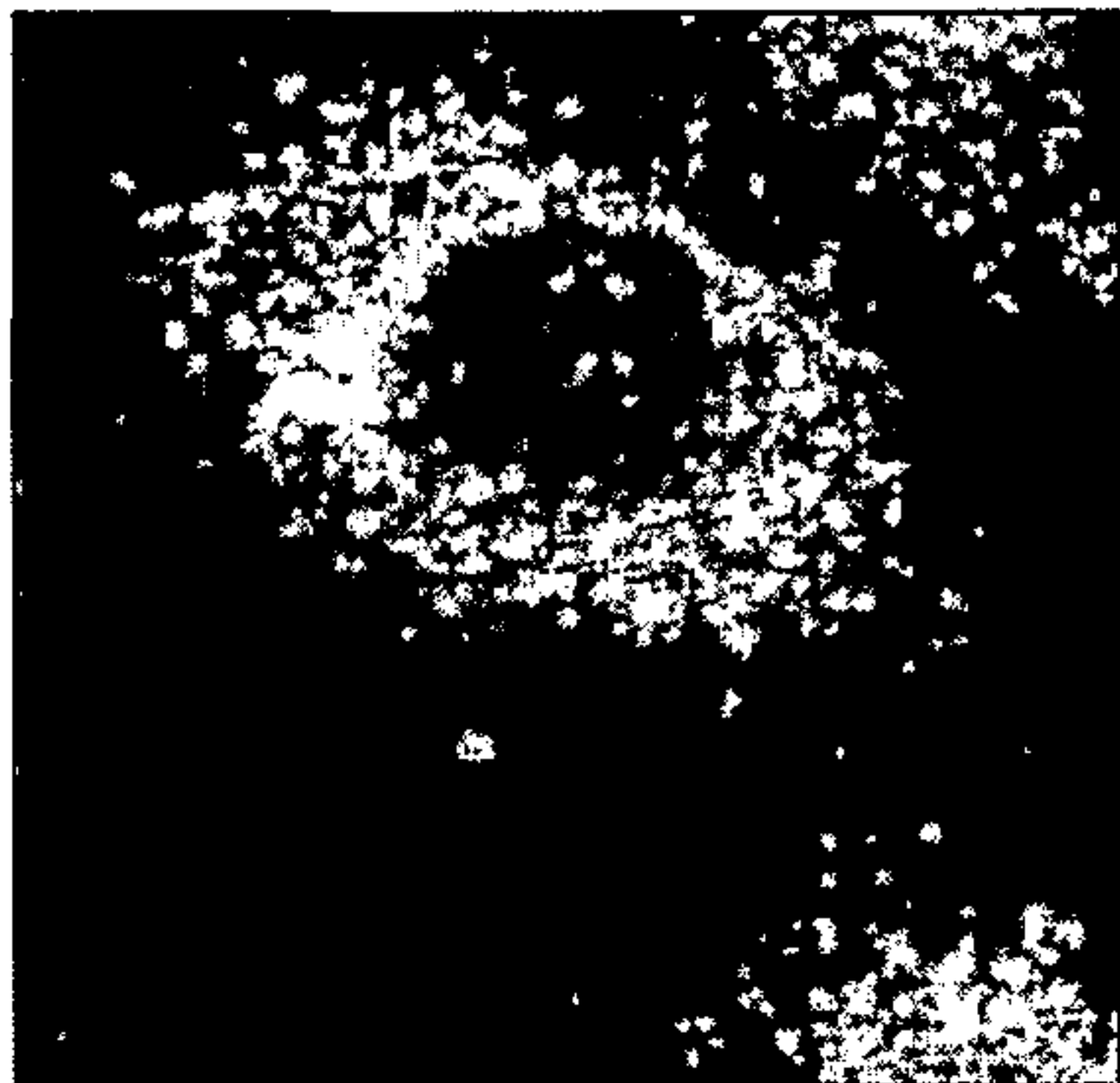
LN-GFP @37 C for 1 hr

FIG. 10E

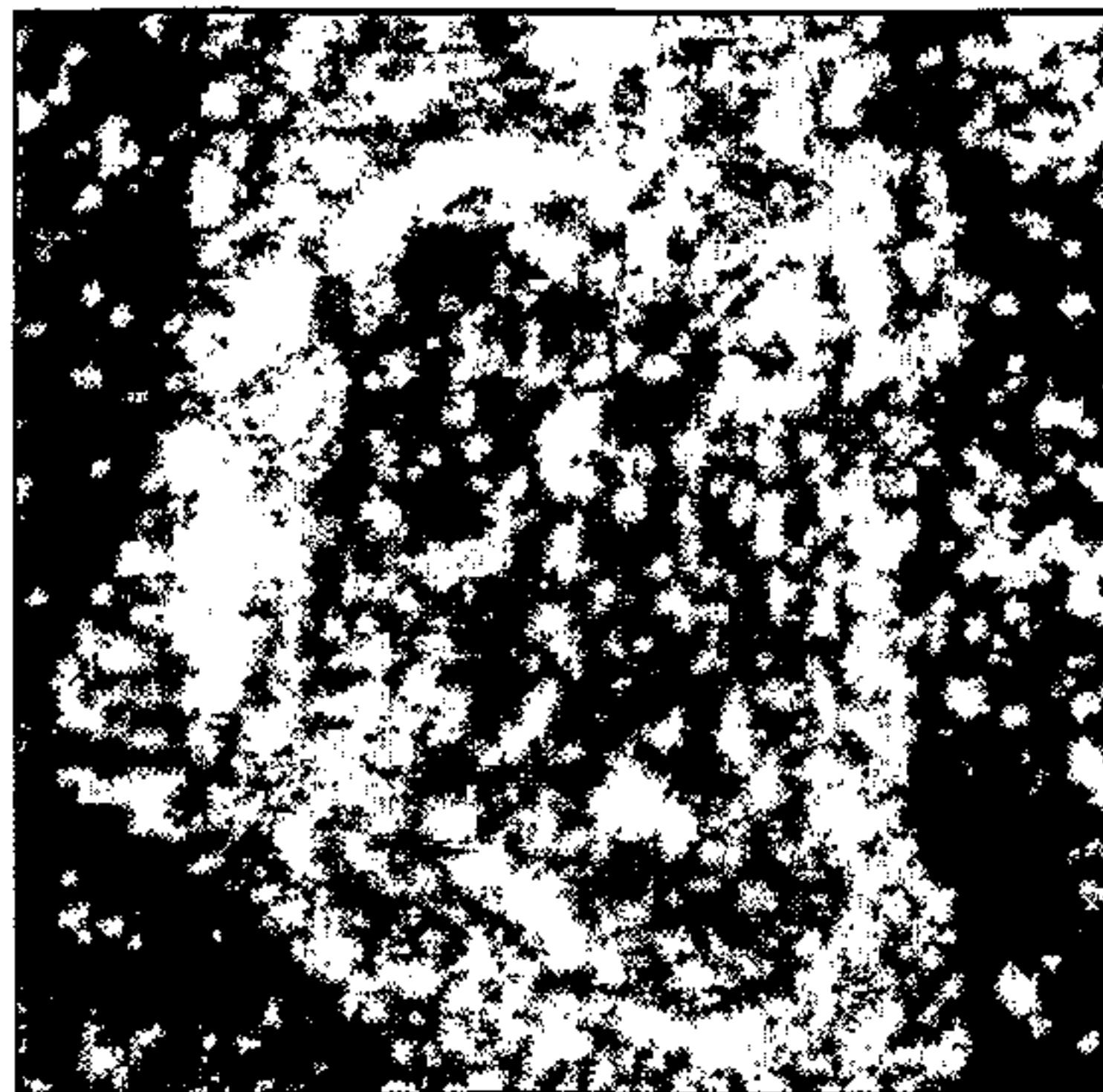
LN-GFP @37 C for 1 hr

FIG. 10F

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LN-GFP @37 C for 1 hr

FIG. 10G

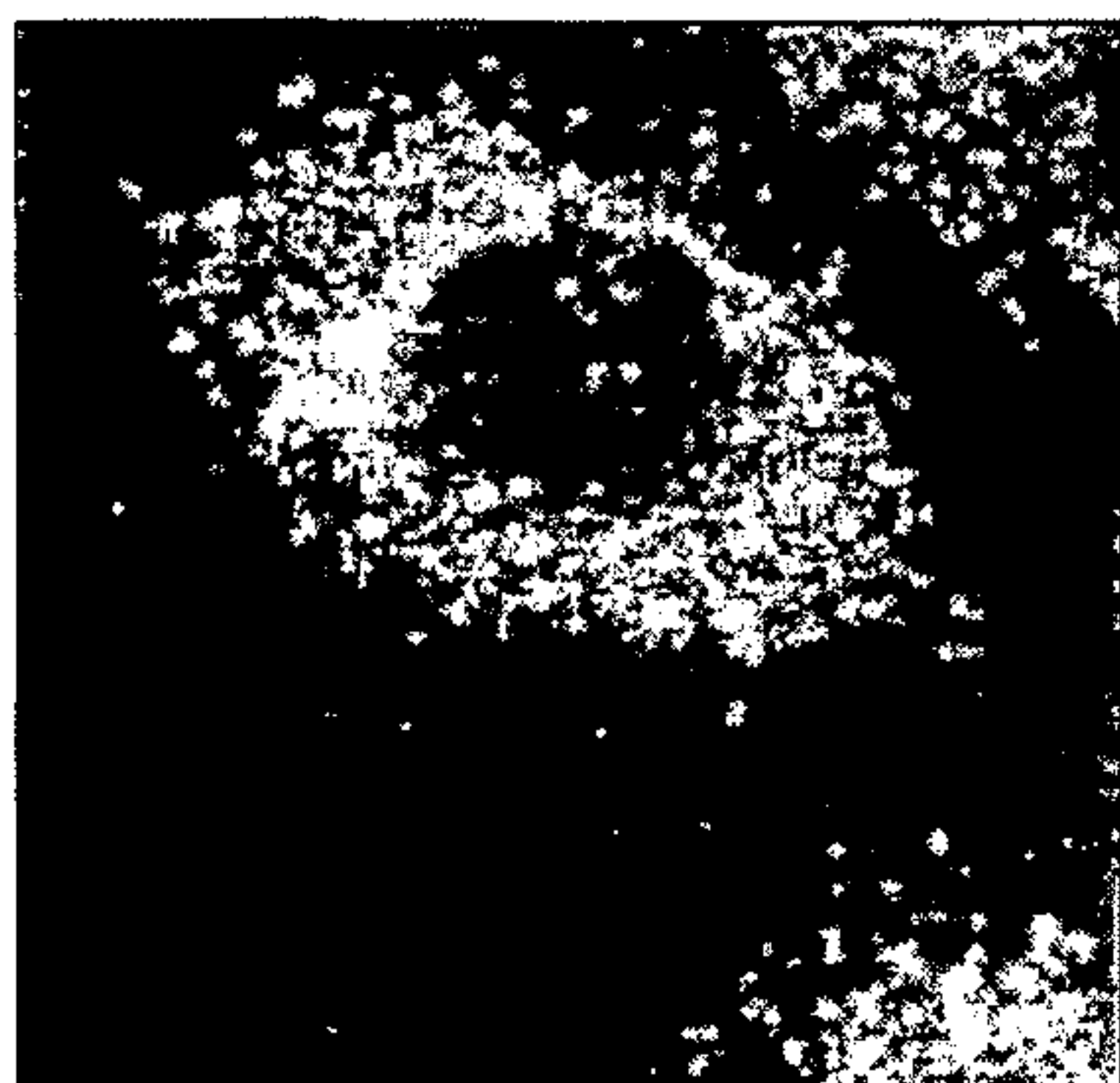
LN-GFP @37 C for 1 hr

FIG. 10H

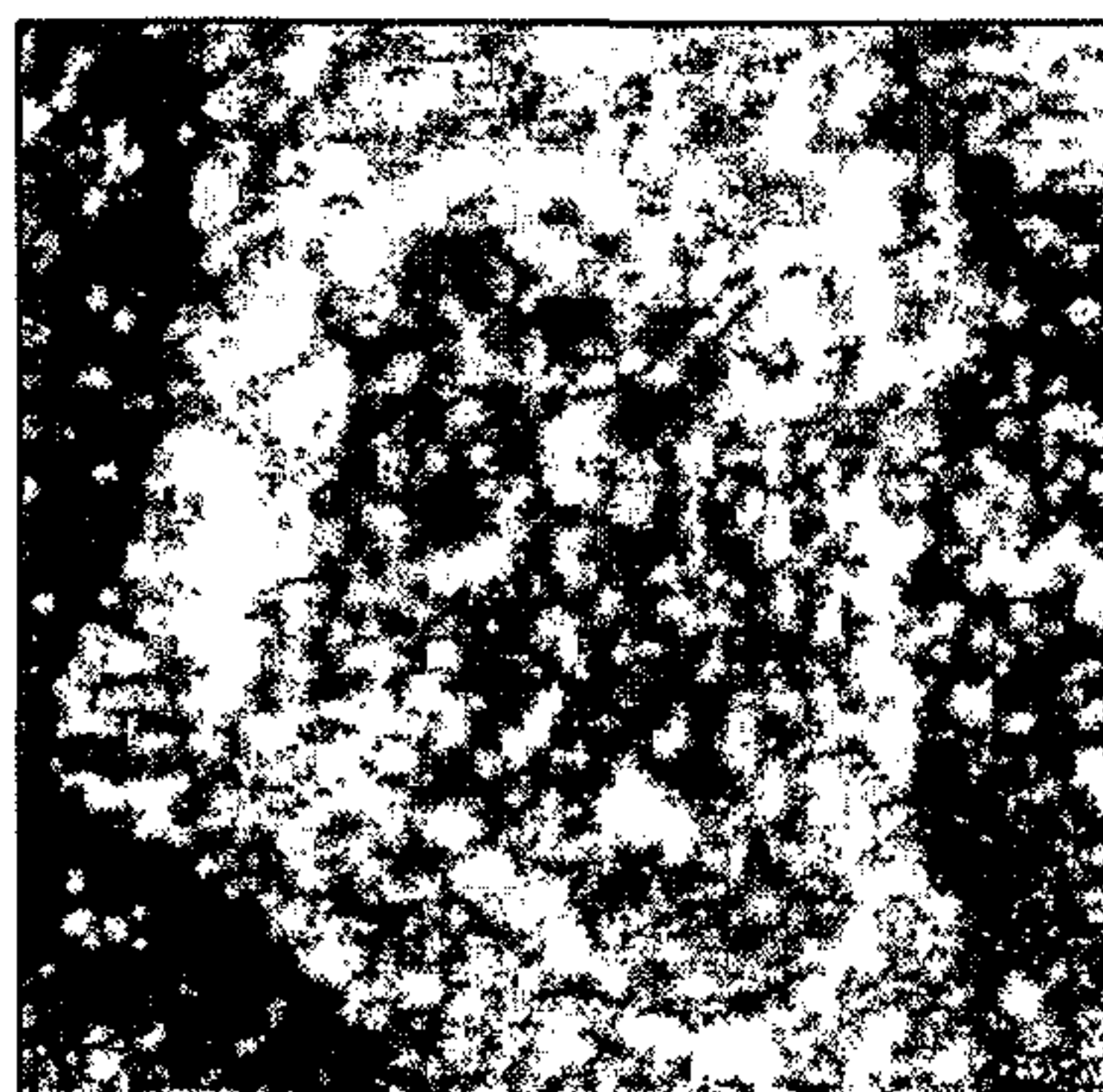
LN-GFP @37 C for 1 hr

FIG. 10I

LN-GFP @37 C for 1 hr

FIG. 10J

LN-GFP @37 C for 1 hr

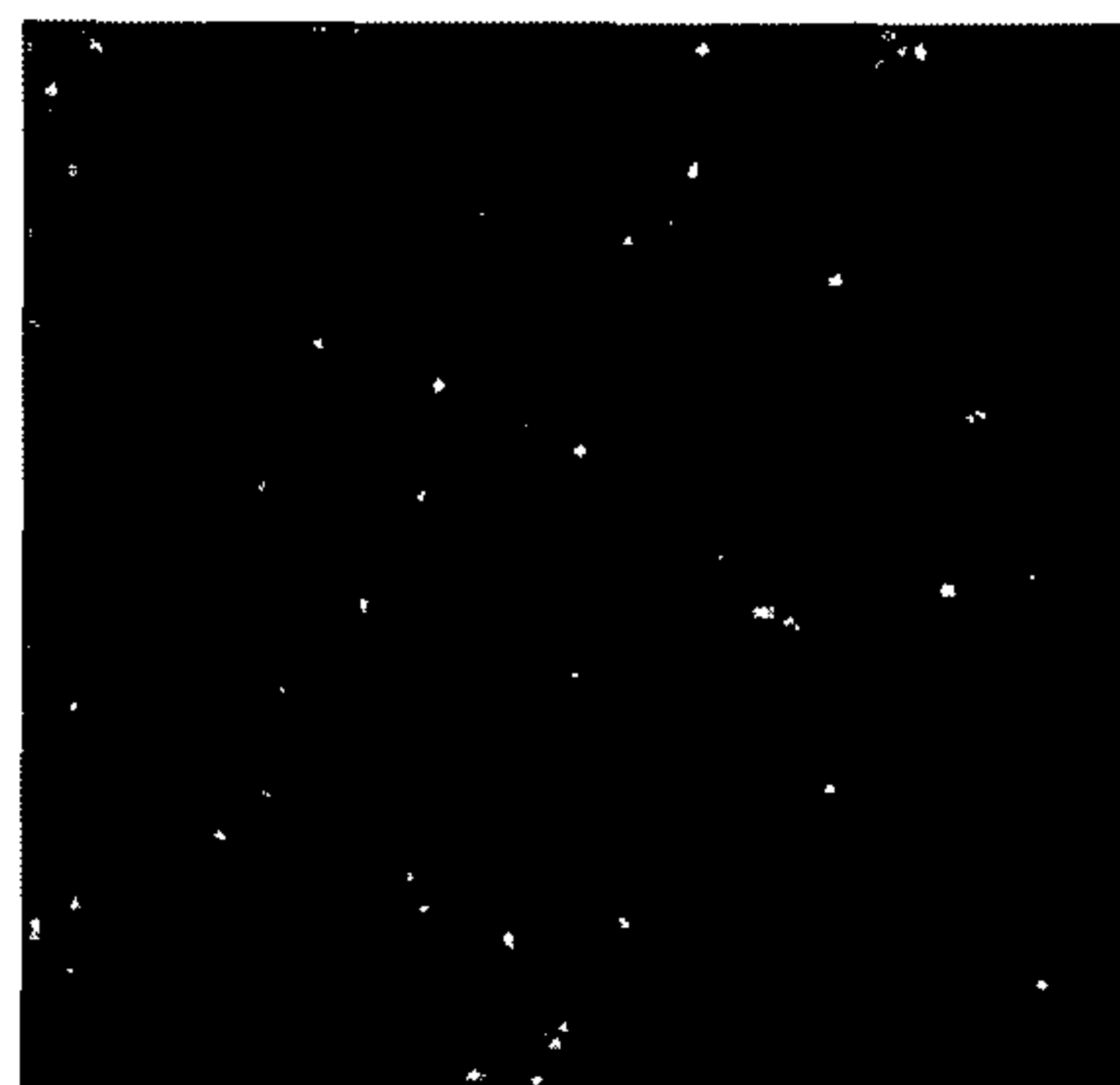
FIG. 10K

LN-GFP @37 C for 1 hr

FIG. 10L

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GFP @37 C for 1 OR 2hr



1h

GFP

FIG. 11A

GFP @37 C for 1 OR 2hr

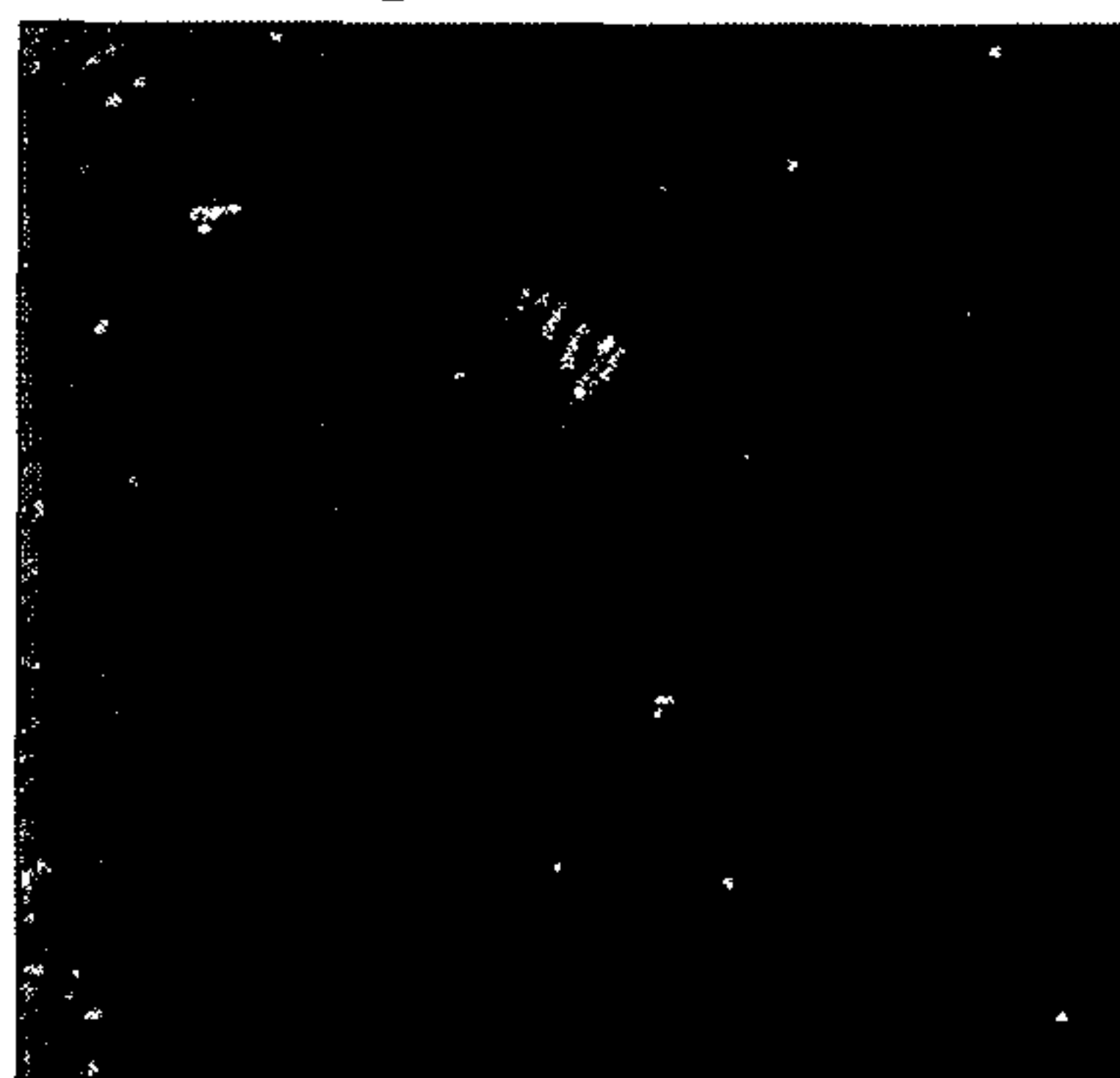


2h

GFP

FIG. 11B

GFP @37 C for 1 OR 2hr



GFP

FIG. 11C

GFP @37 C for 1 OR 2hr

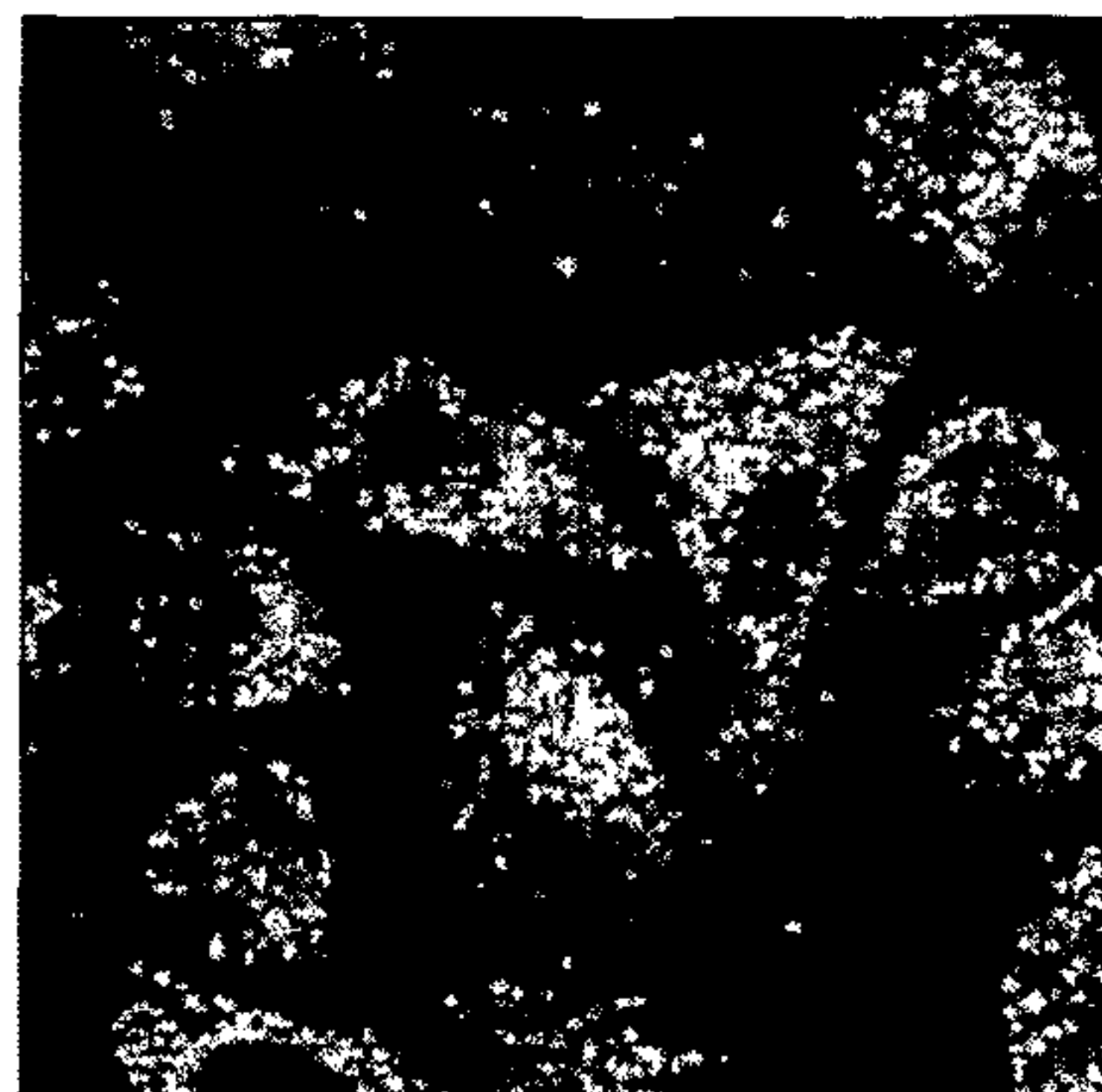


1h

Transferin

FIG. 11D

GFP @37 C for 1 OR 2hr



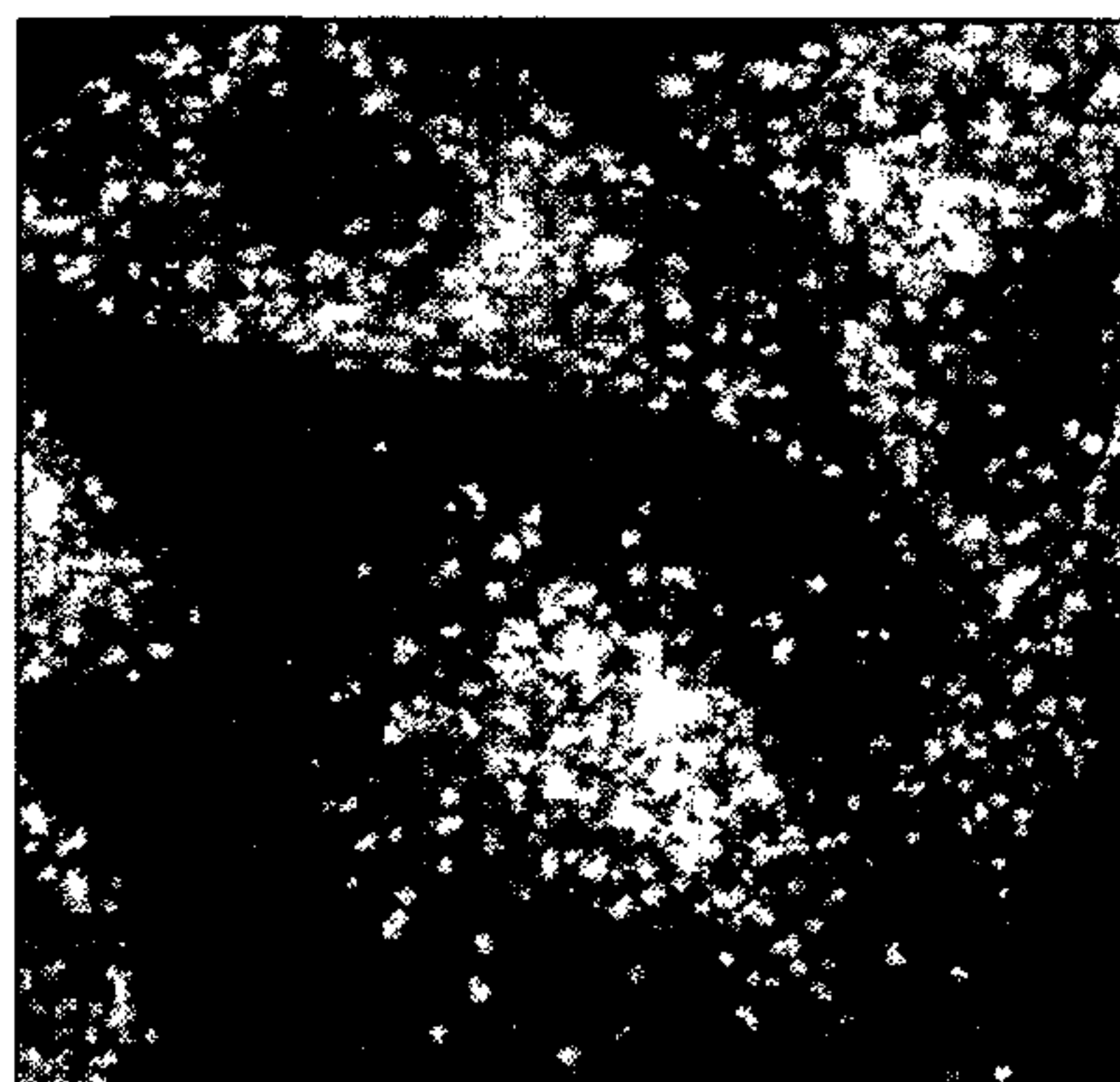
2h

Transferin

FIG. 11E

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GFP @37 C for 1 OR 2hr



Transferin

FIG. 11F

GFP @37 C for 1 OR 2hr

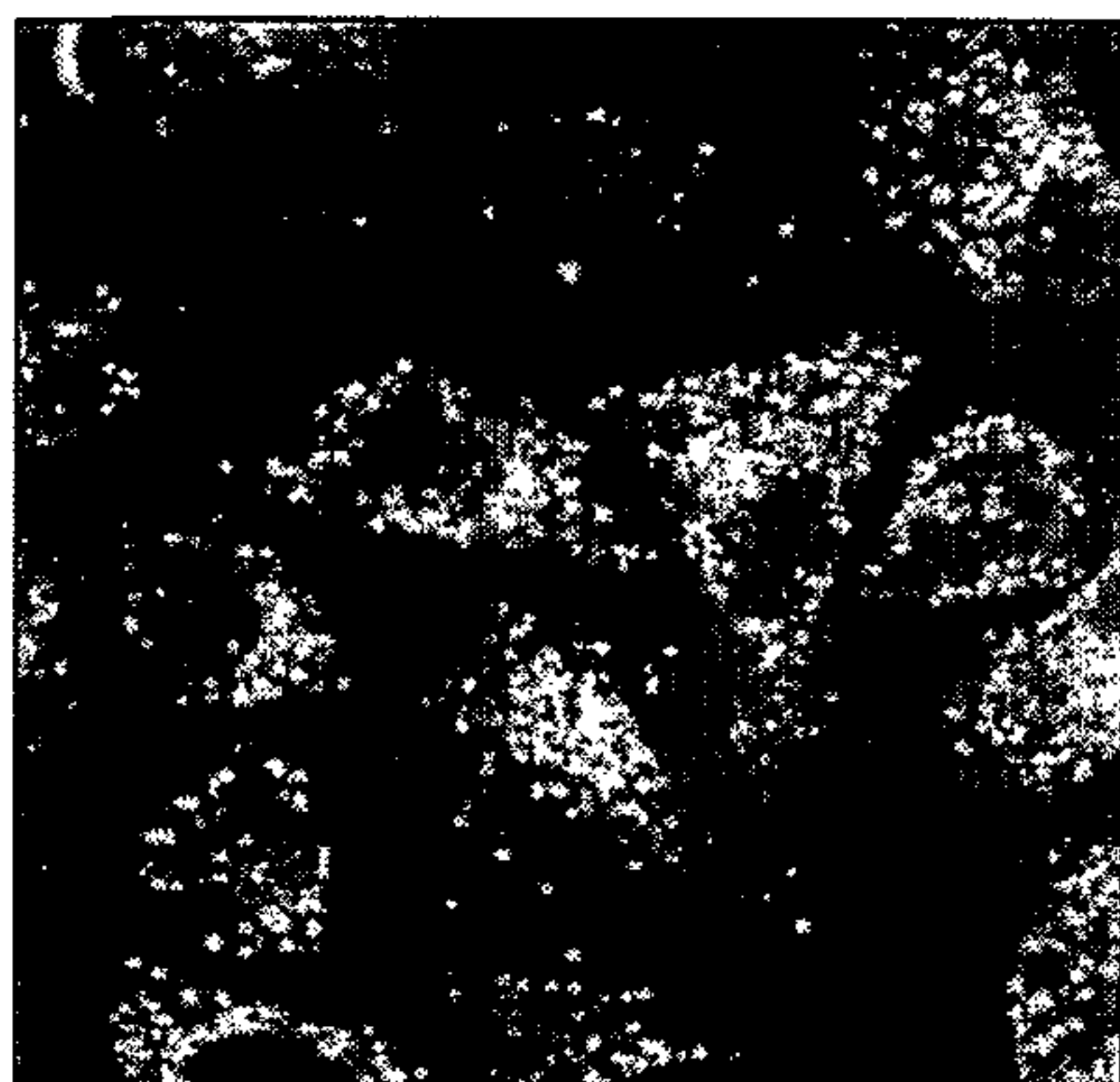


1h

Merge

FIG. 11G

GFP @37 C for 1 OR 2hr

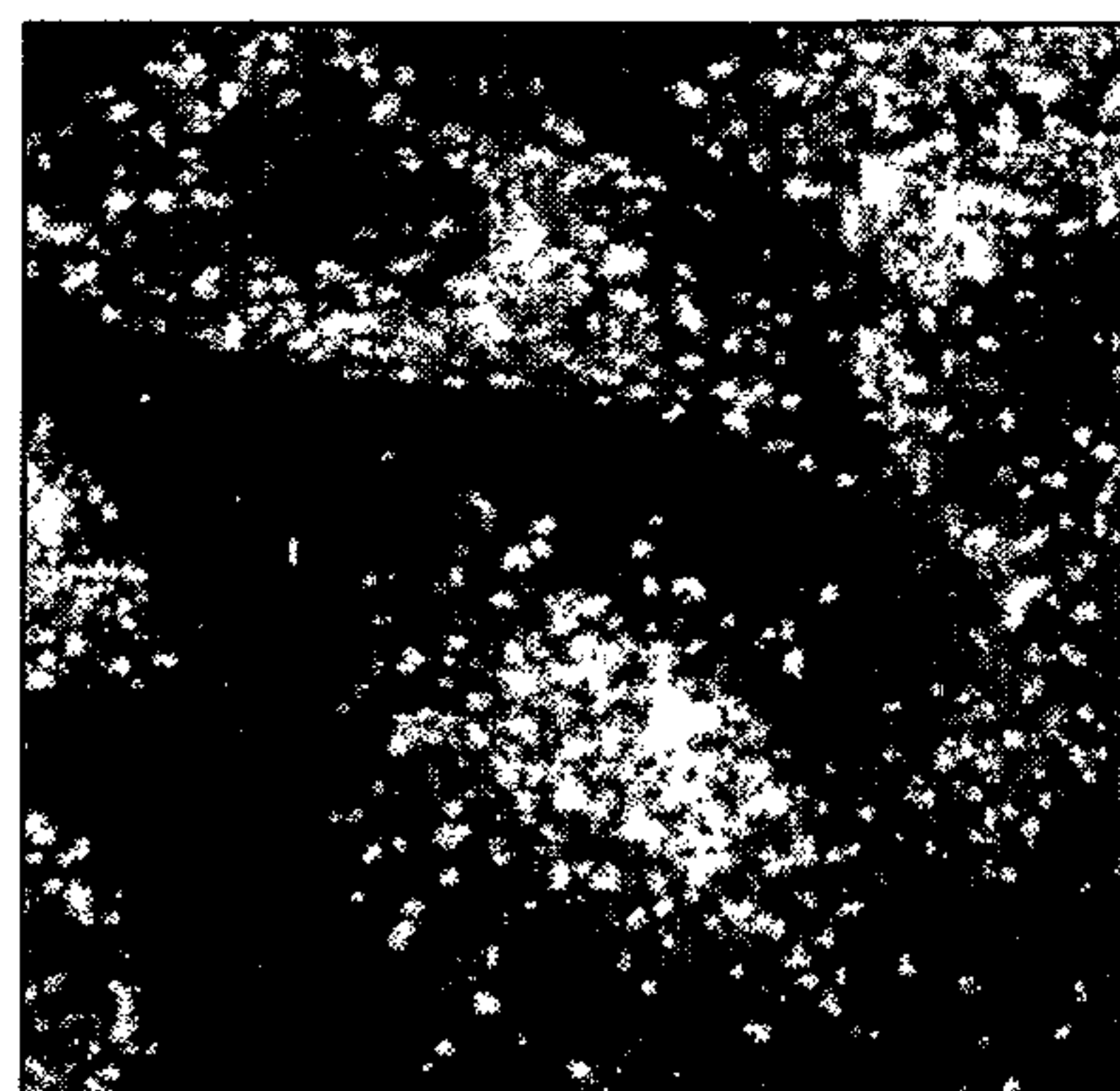


2h

Merge

FIG. 11H

GFP @37 C for 1 OR 2hr



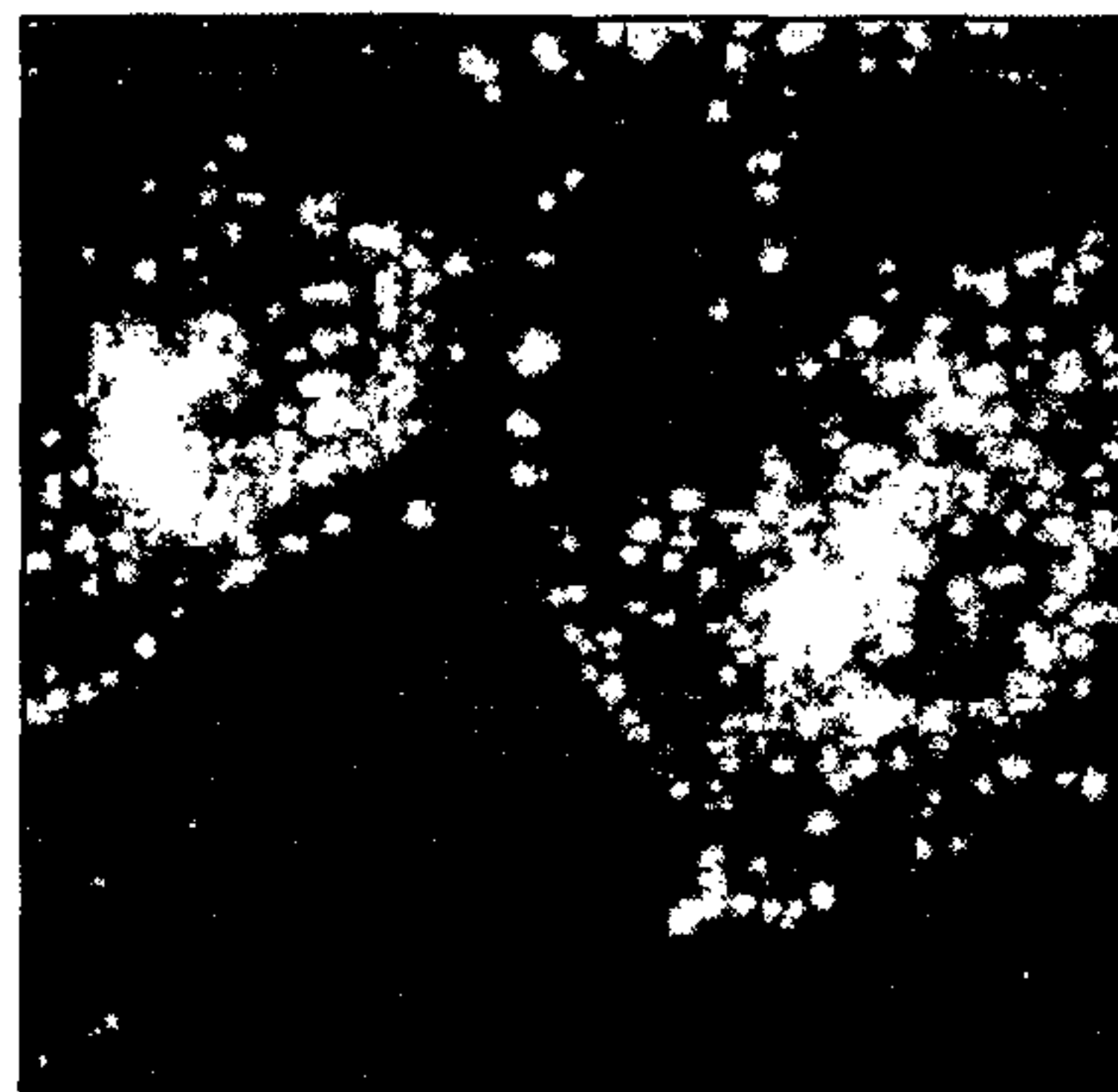
Merge

FIG. 11I

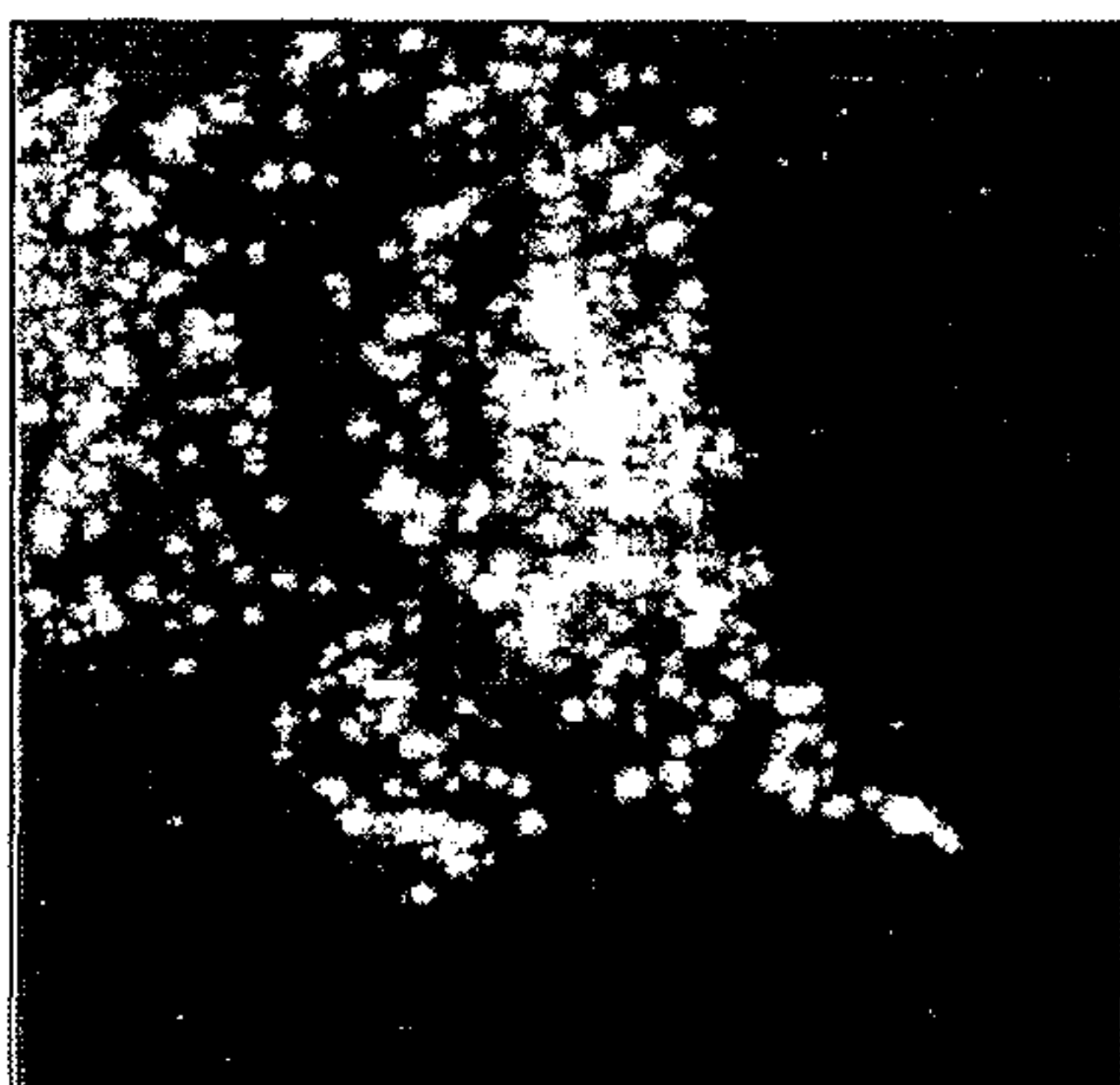
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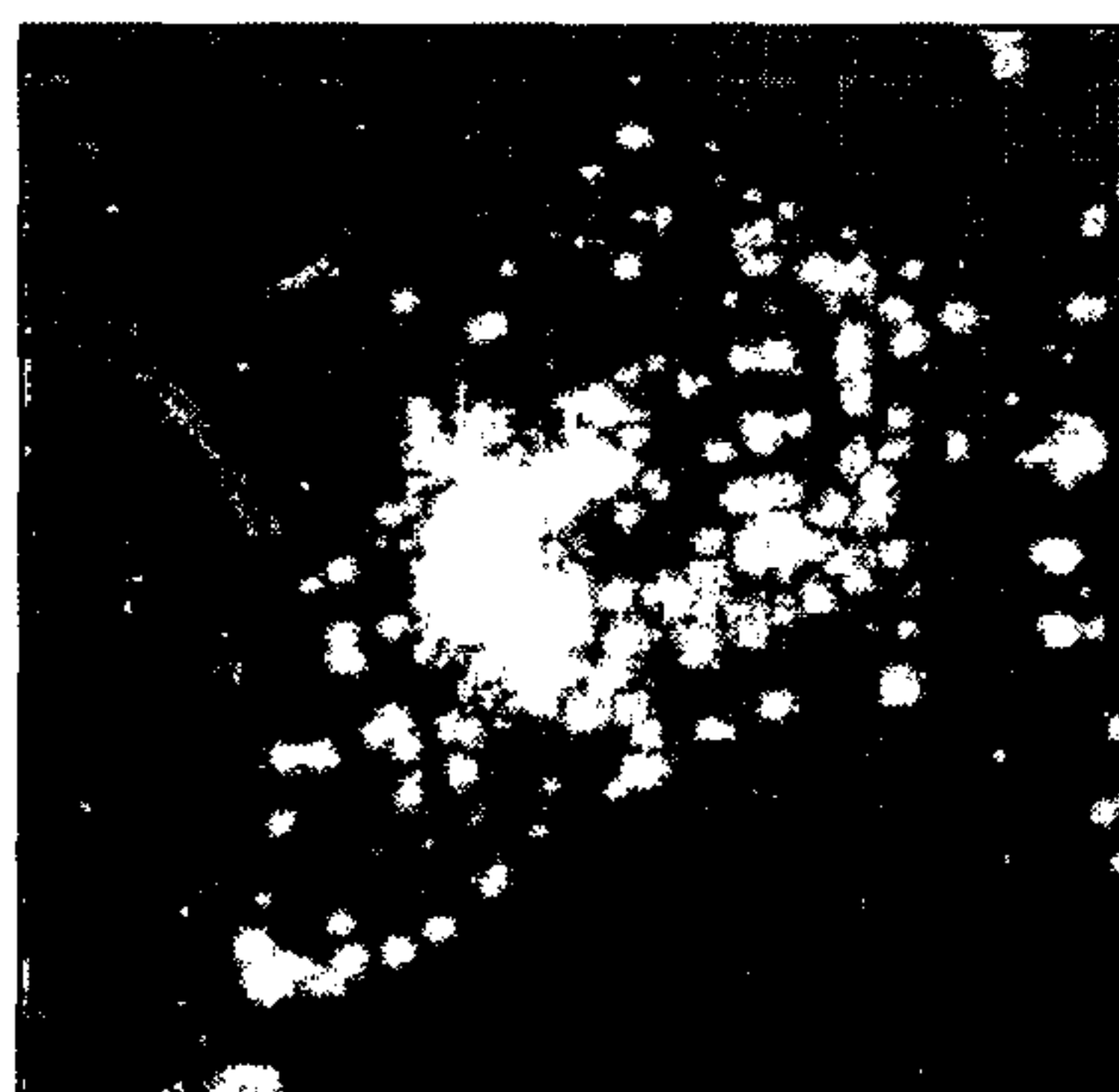
Lamp-2

FIG. 12A

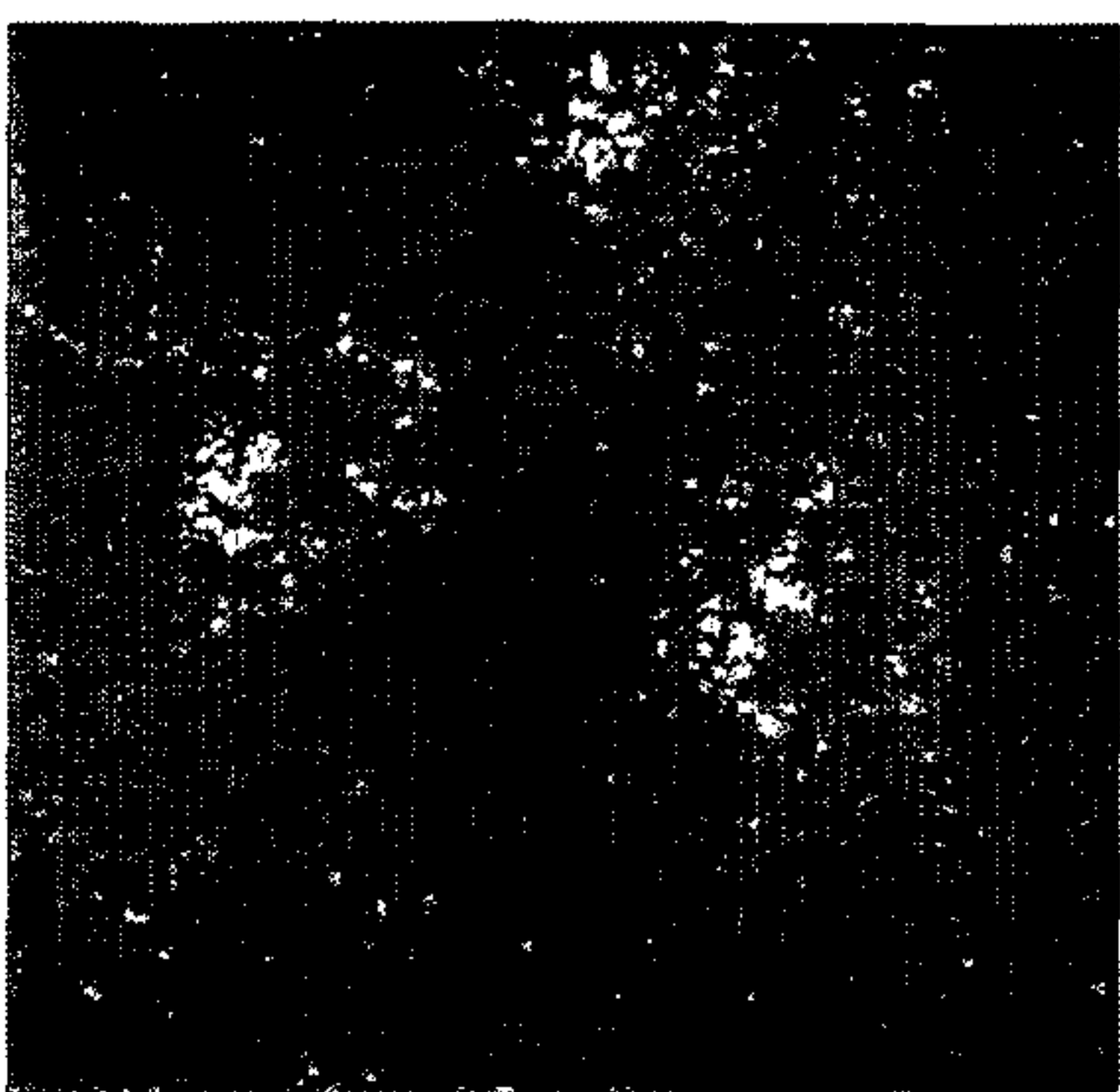
Lamp-2

FIG. 12B

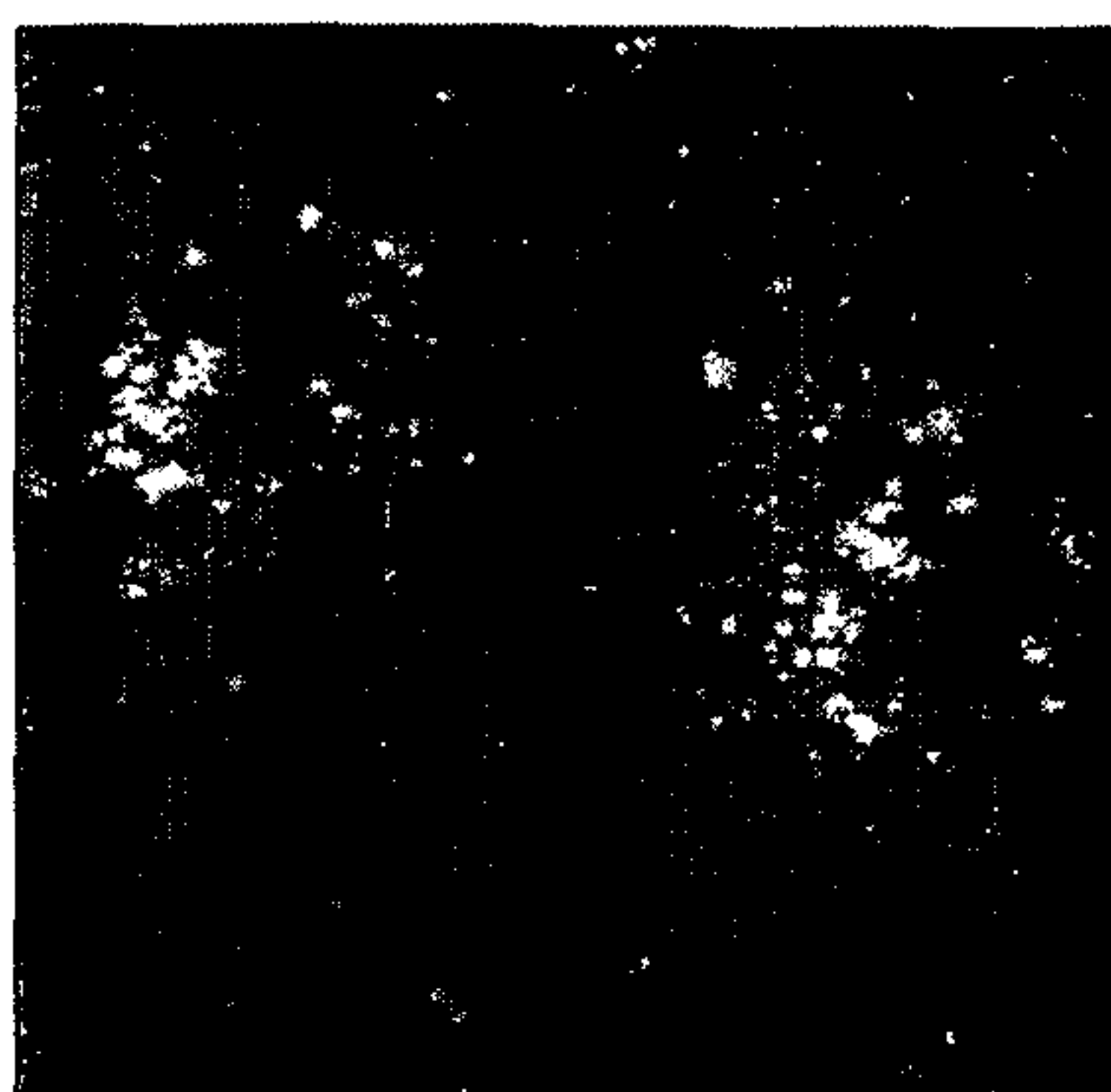
Lamp-2

FIG. 12C

Lamp-2

FIG. 12D

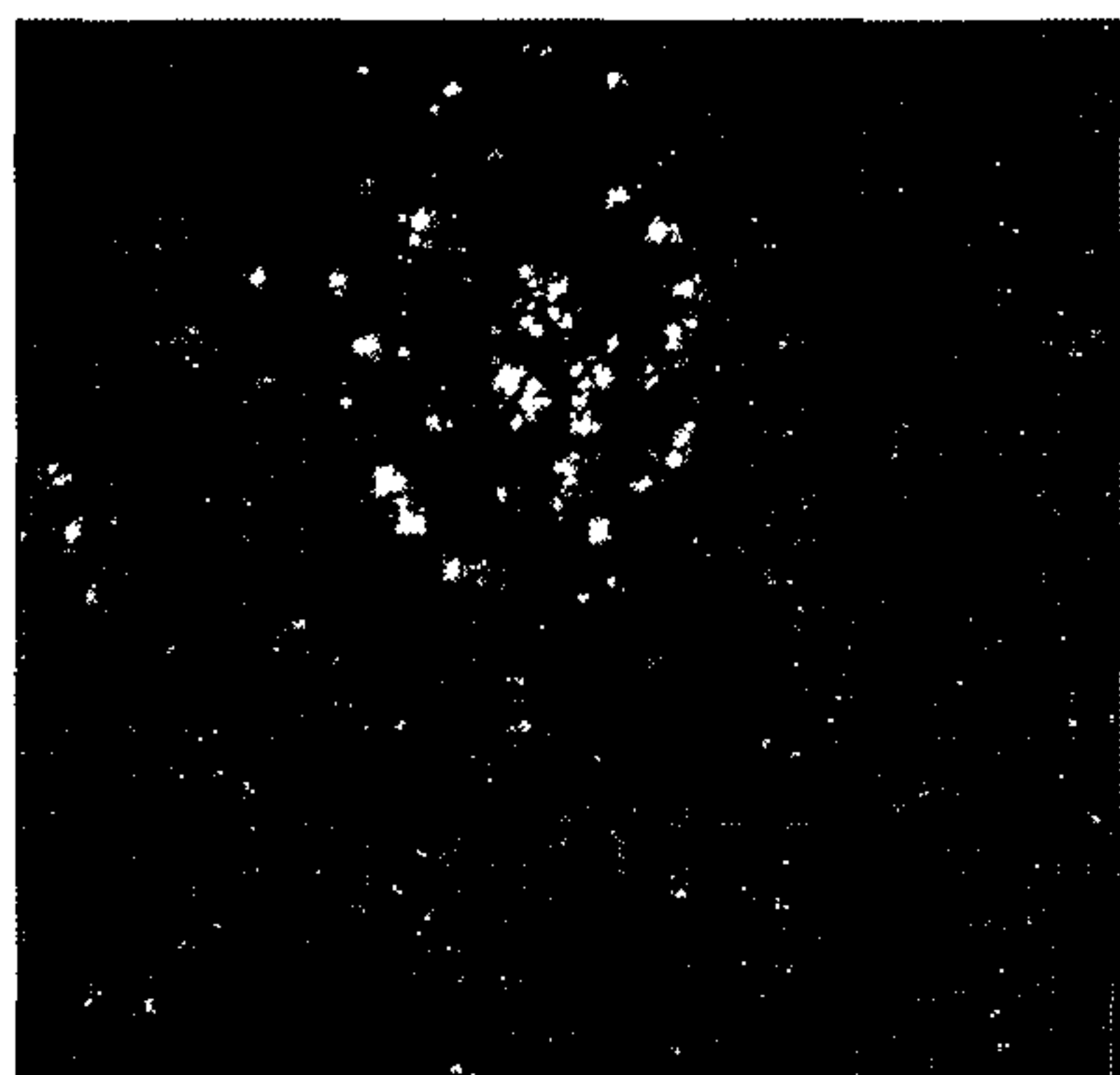
LN-GFP

FIG. 12E

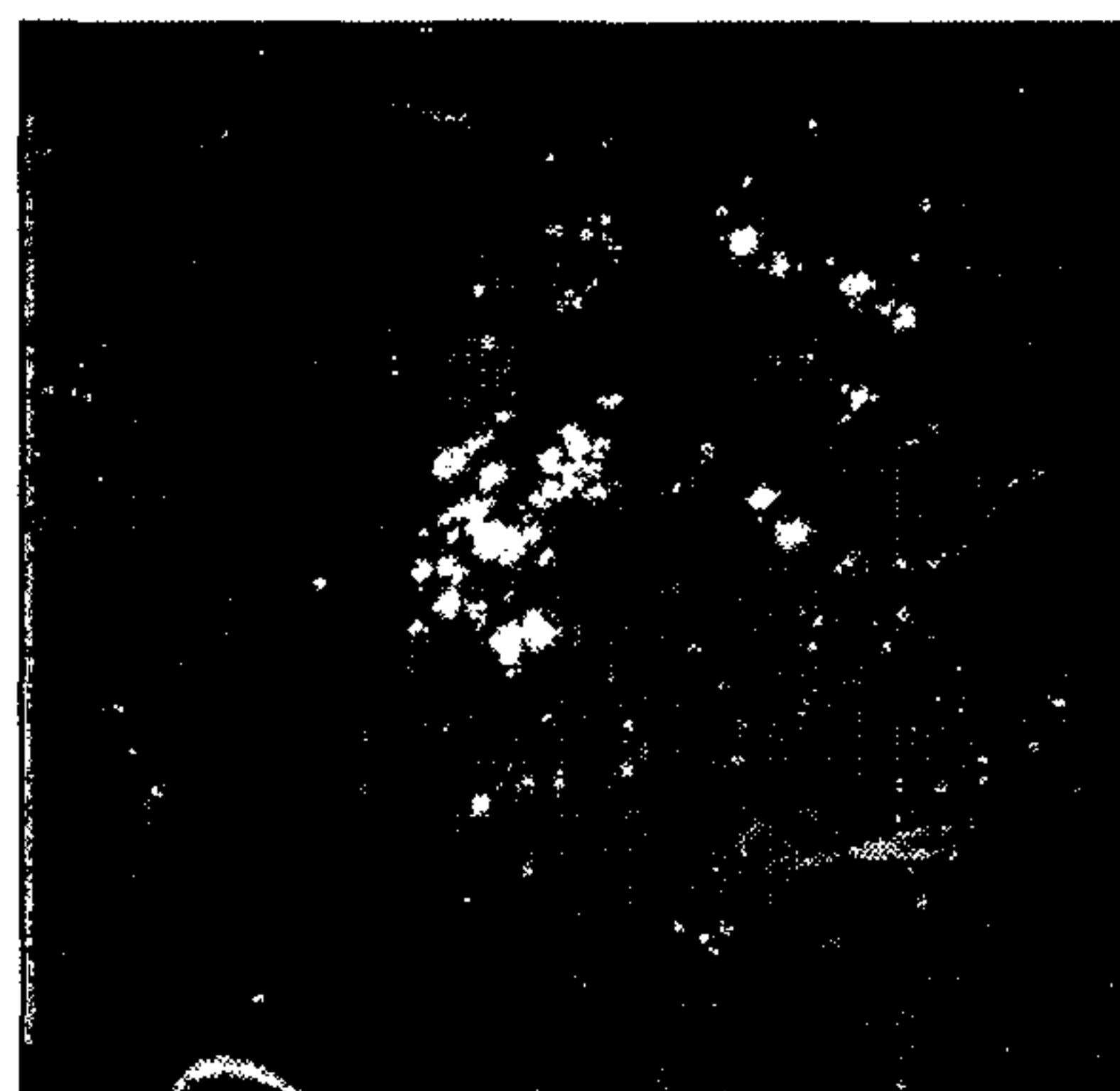
LN-GFP

FIG. 12F

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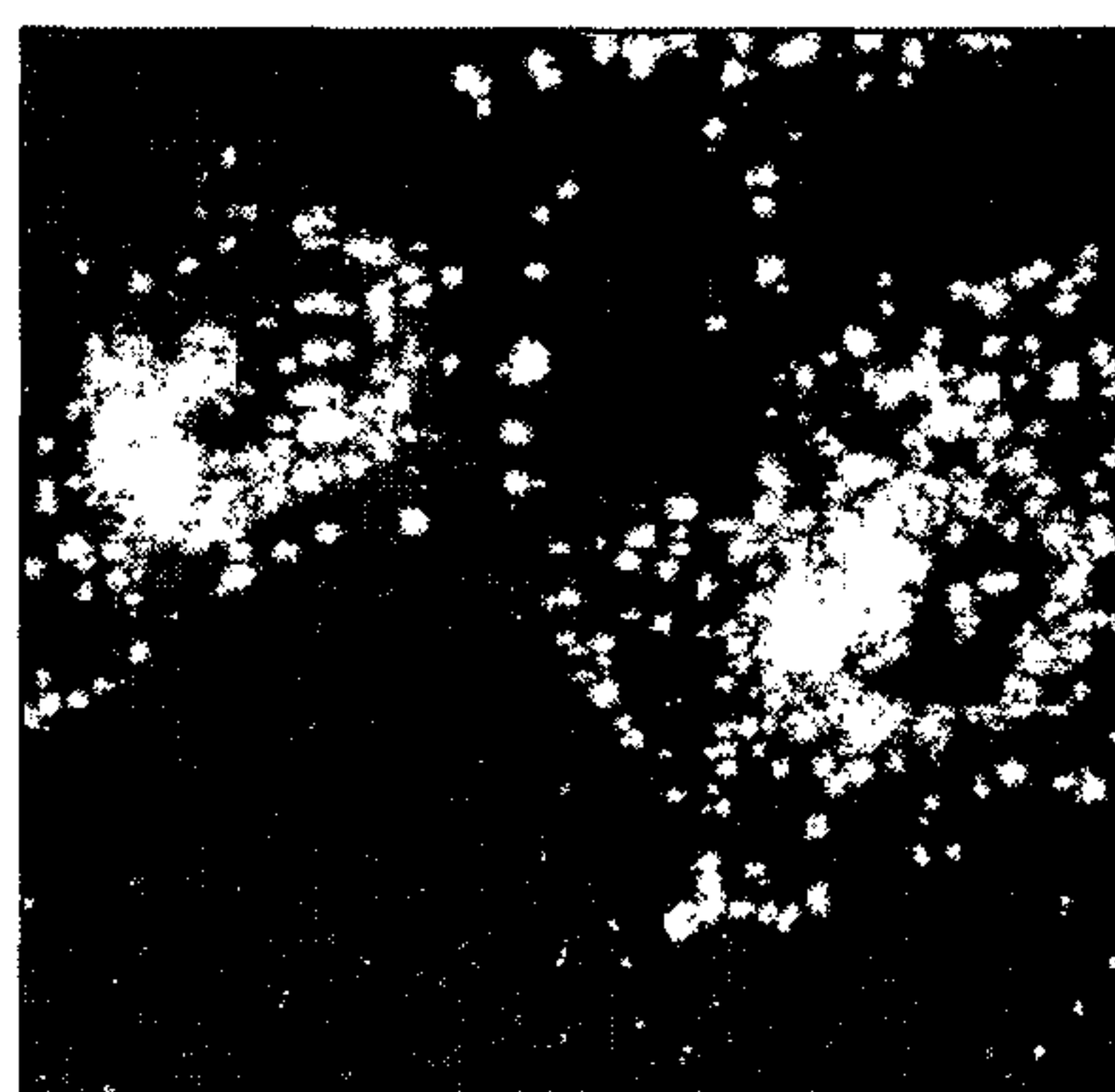
LN-GFP

FIG. 12G

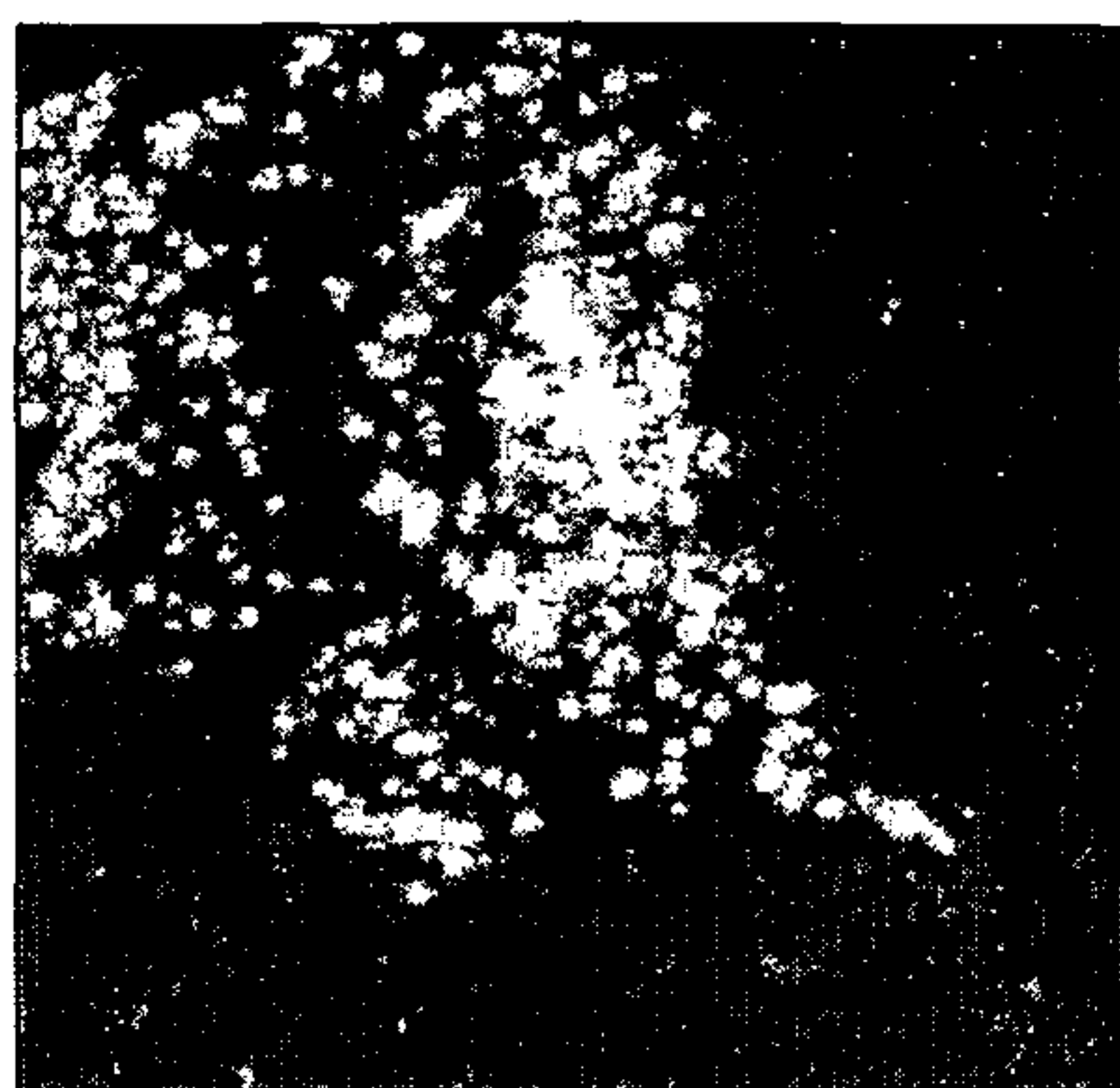
LN-GFP

FIG. 12H

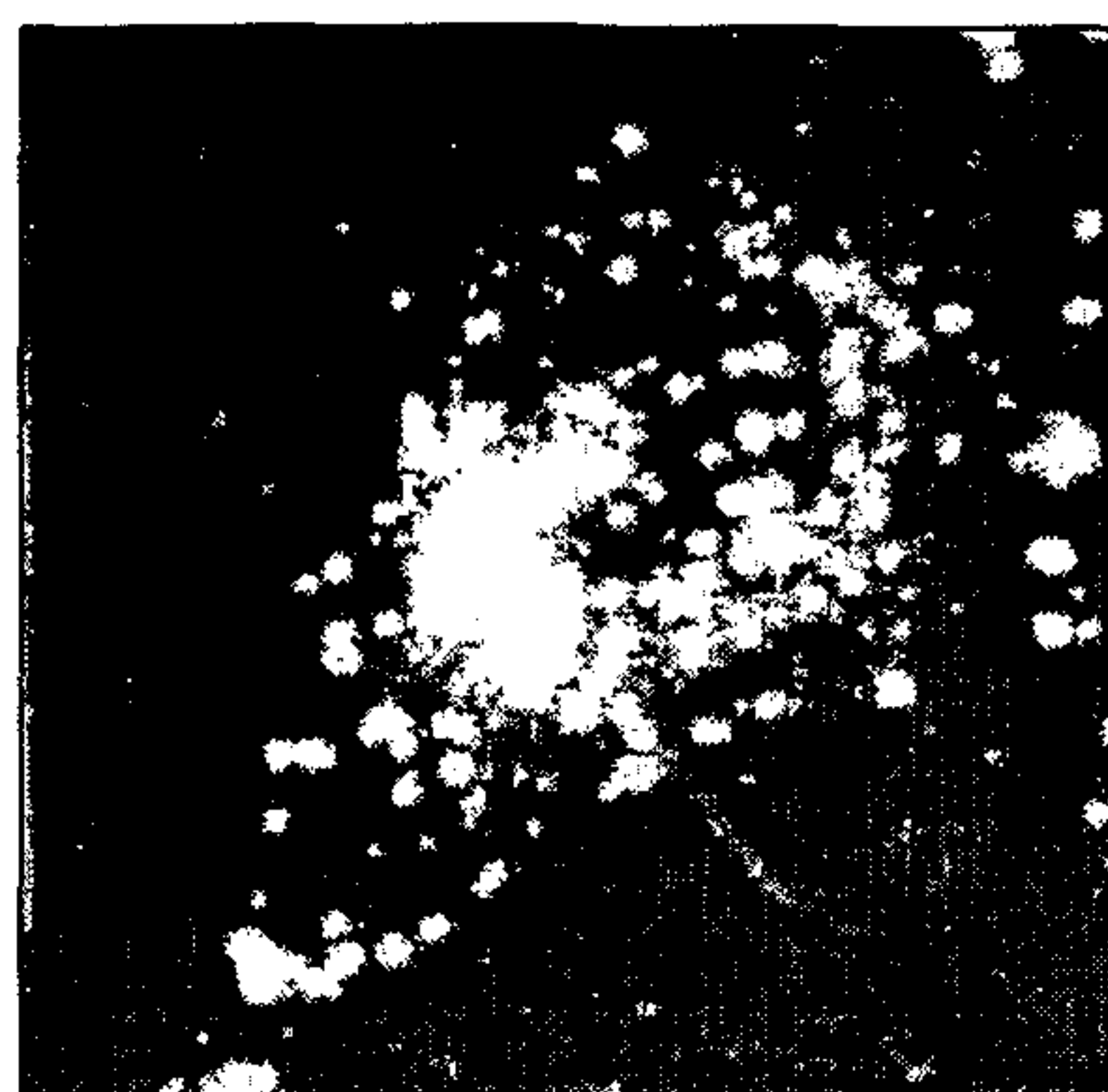
Merge

FIG. 12I

Merge

FIG. 12J

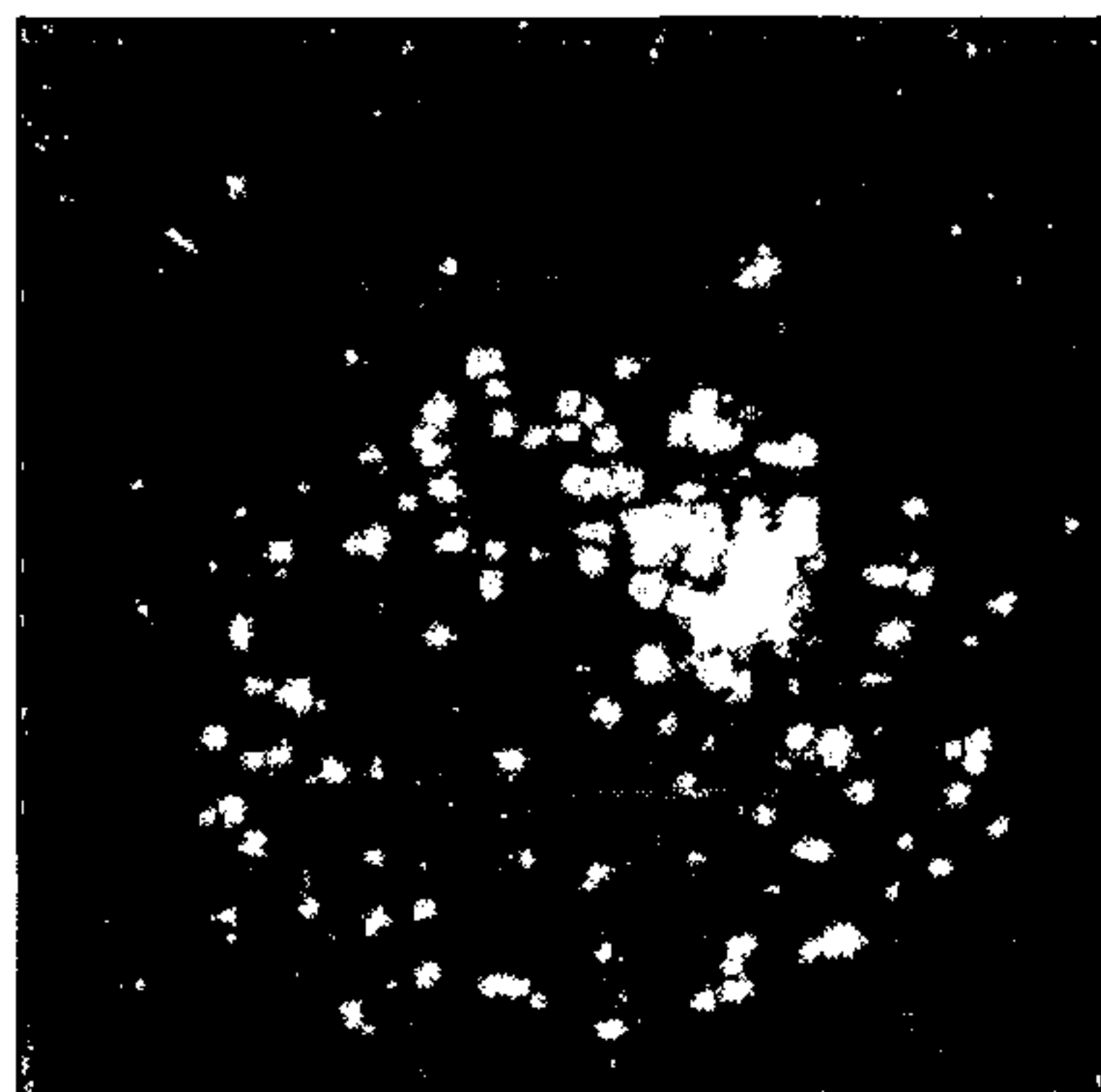
Merge

FIG. 12K

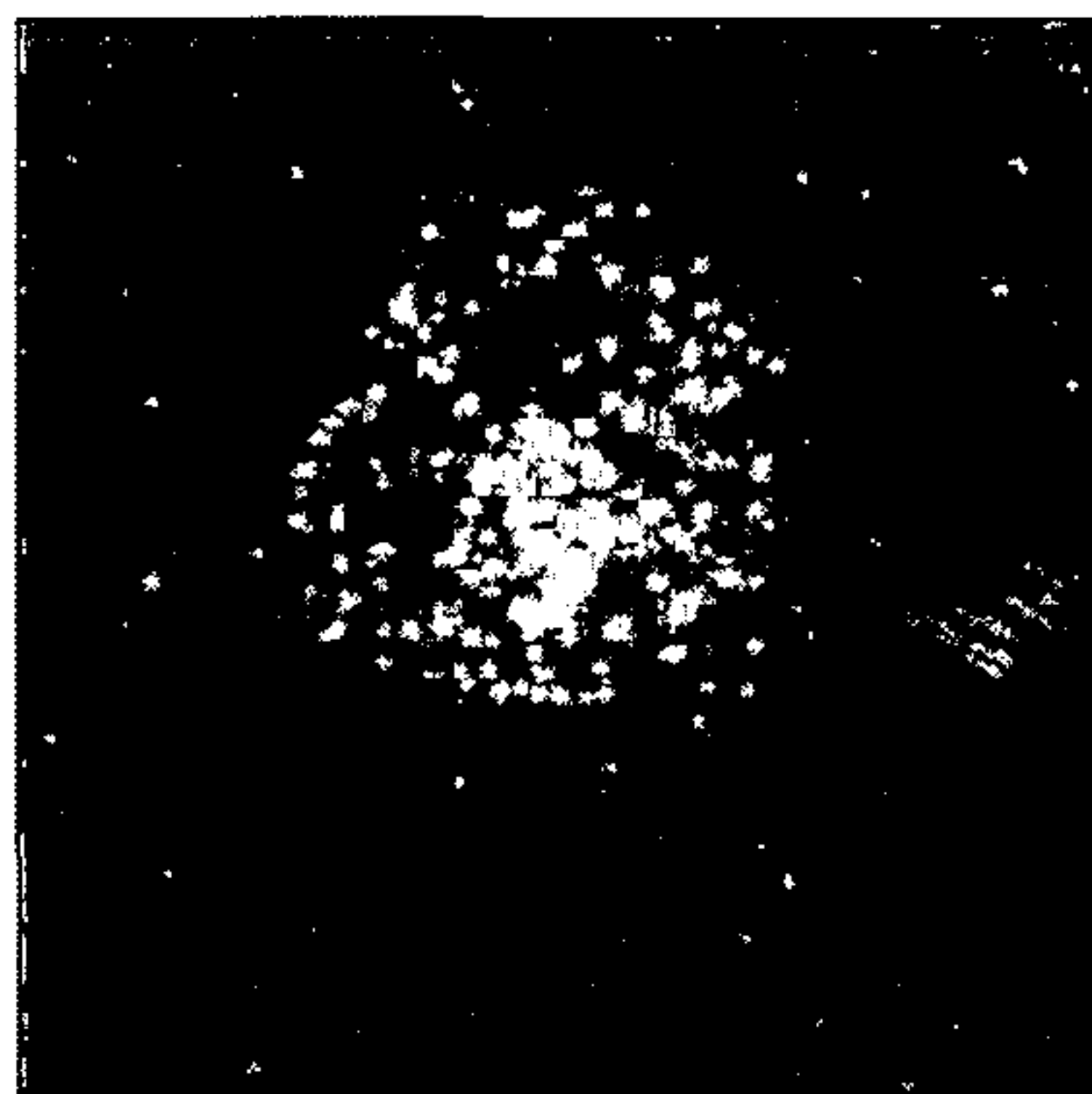
Merge

FIG. 12L

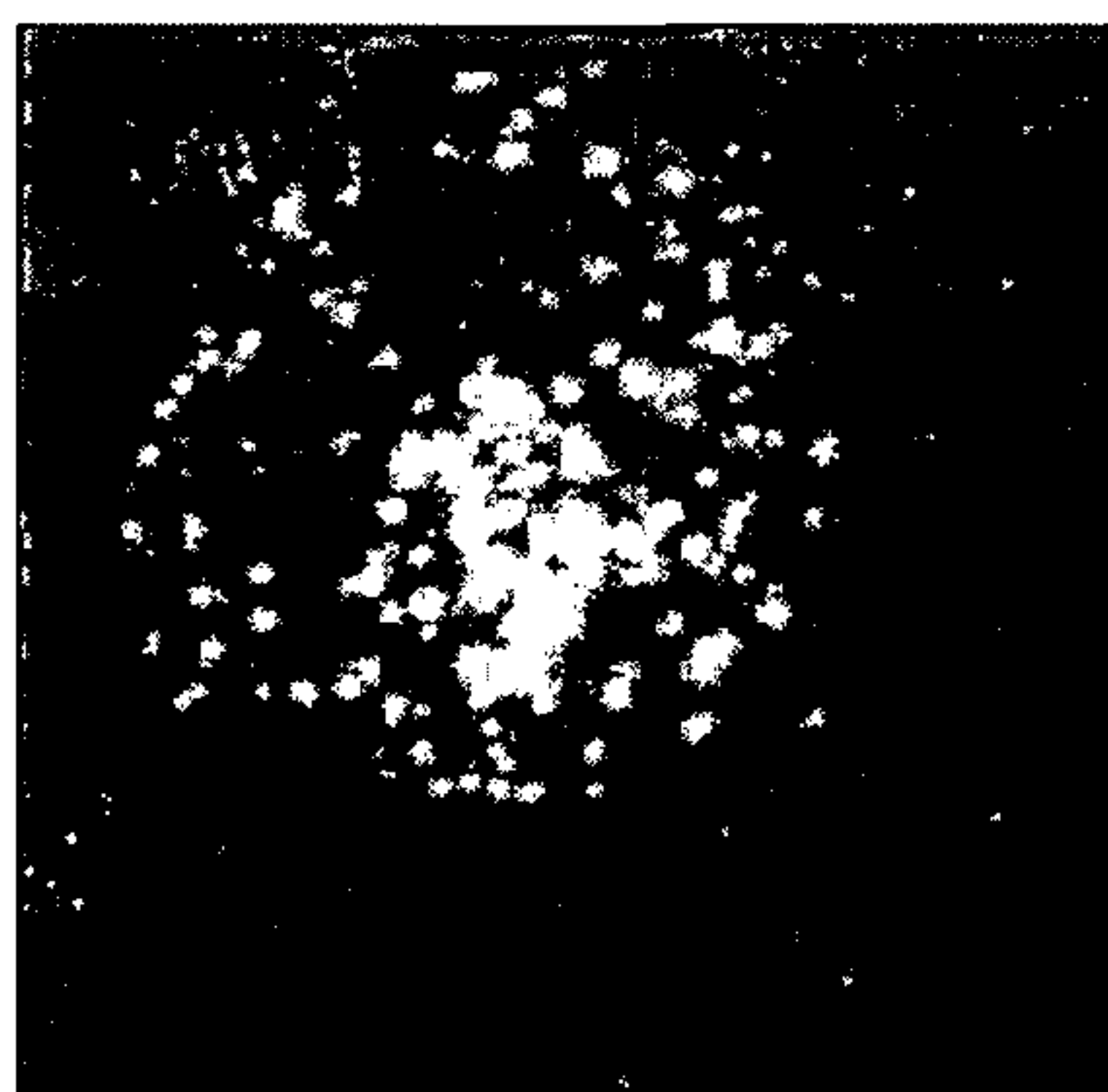
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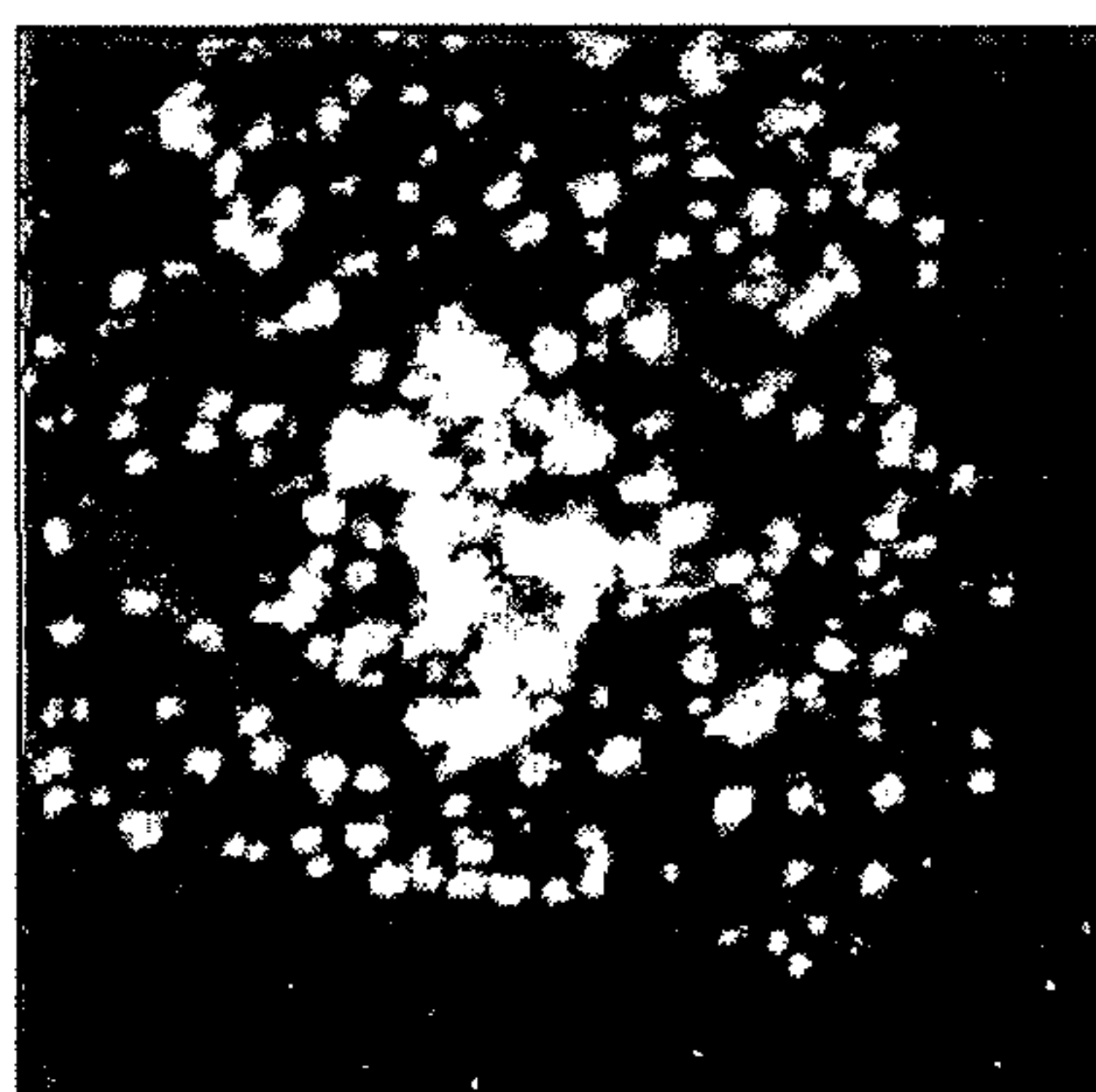
EEA-1

FIG. 13A

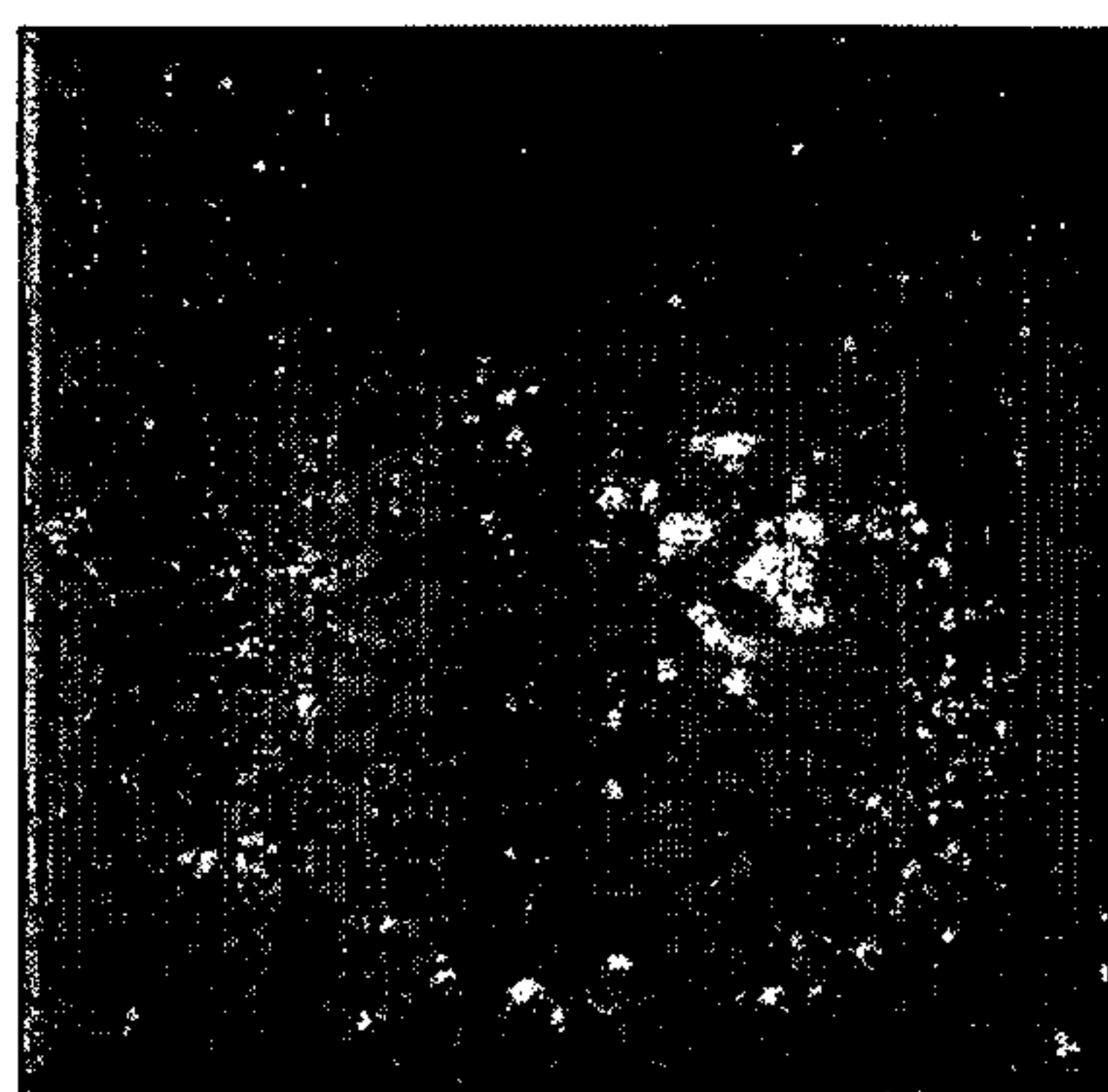
EEA-1

FIG. 13B

EEA-1

FIG. 13C

EEA-1

FIG. 13D

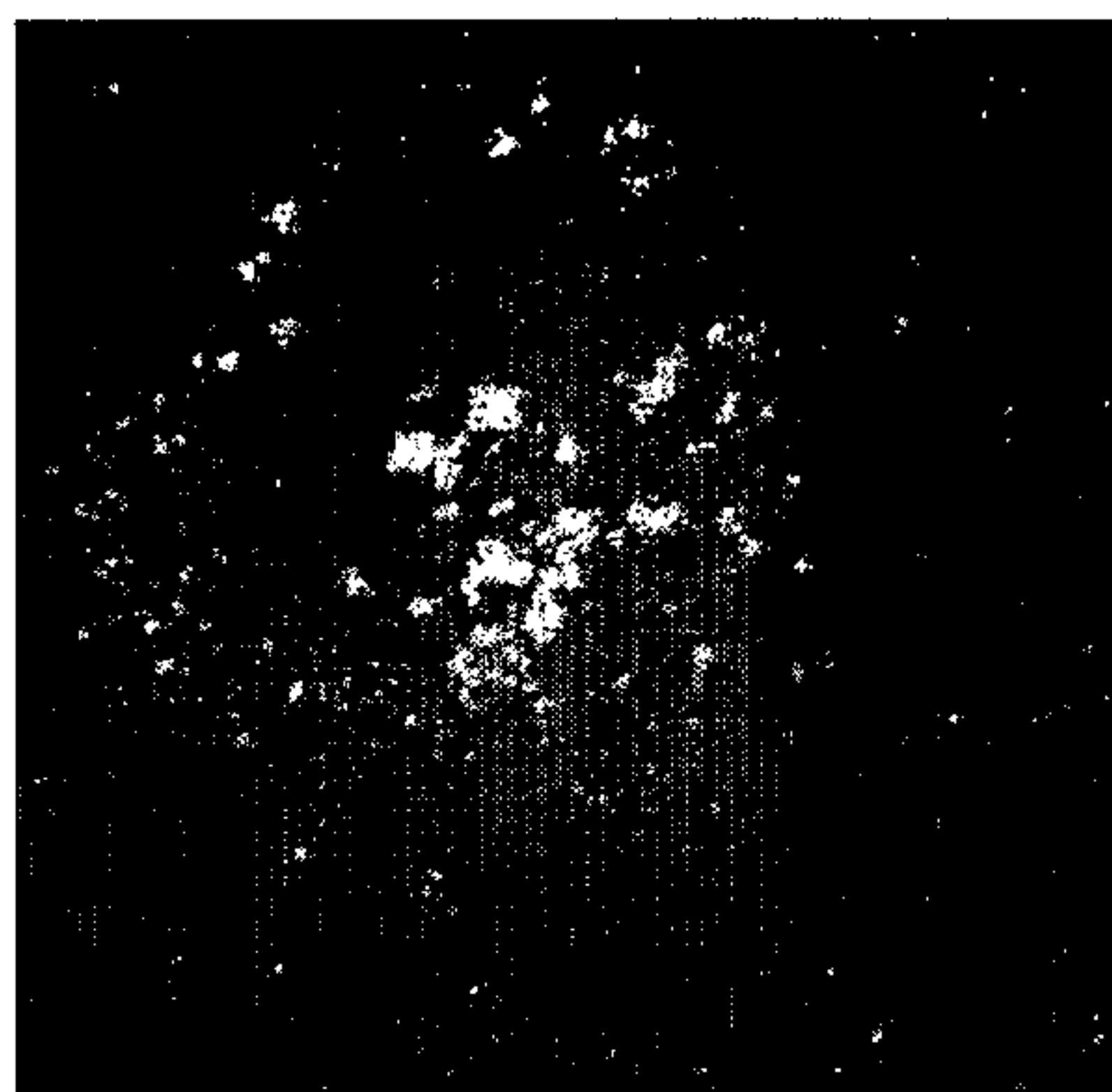
LN-GFP

FIG. 13E

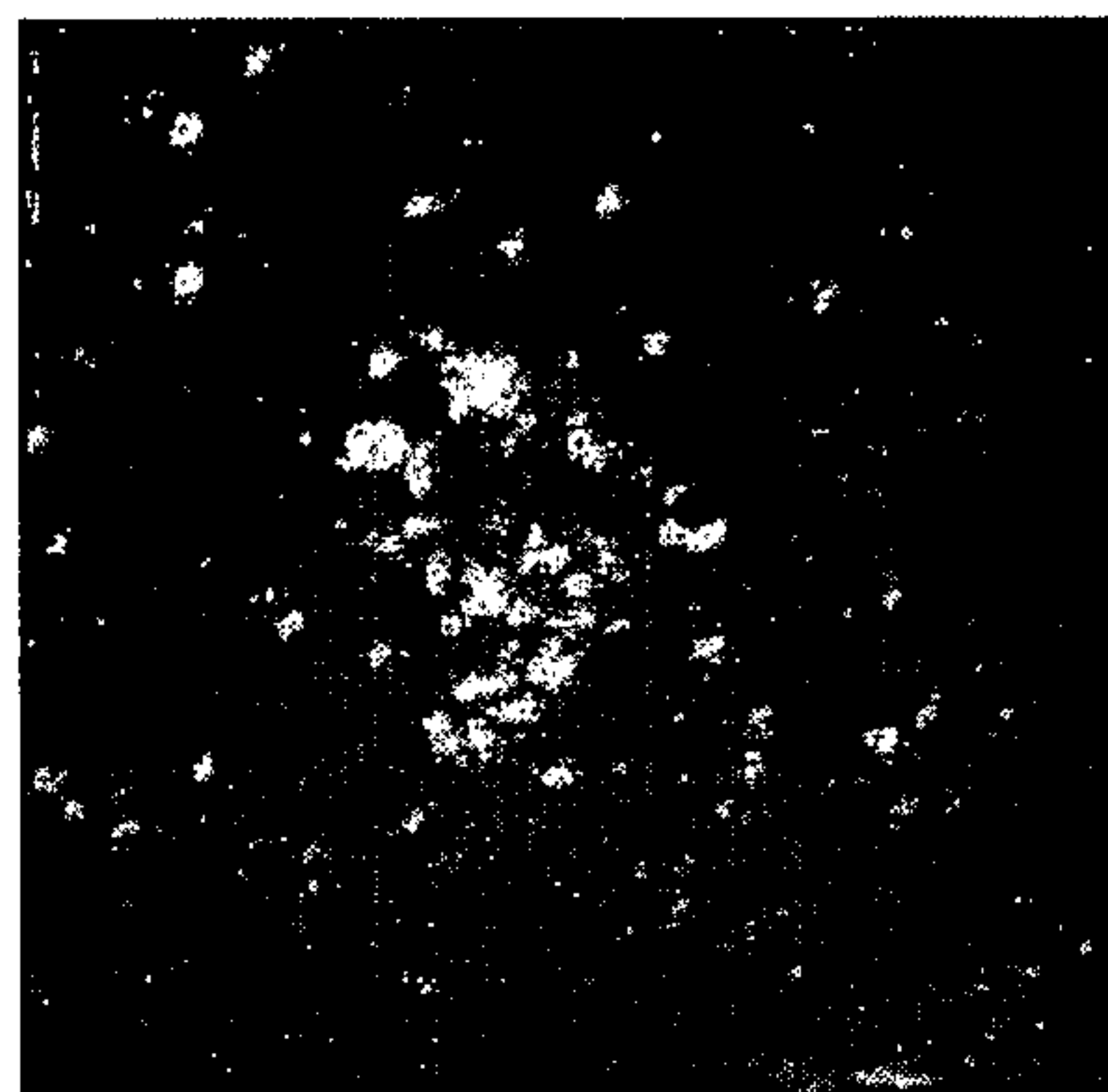
LN-GFP

FIG. 13F

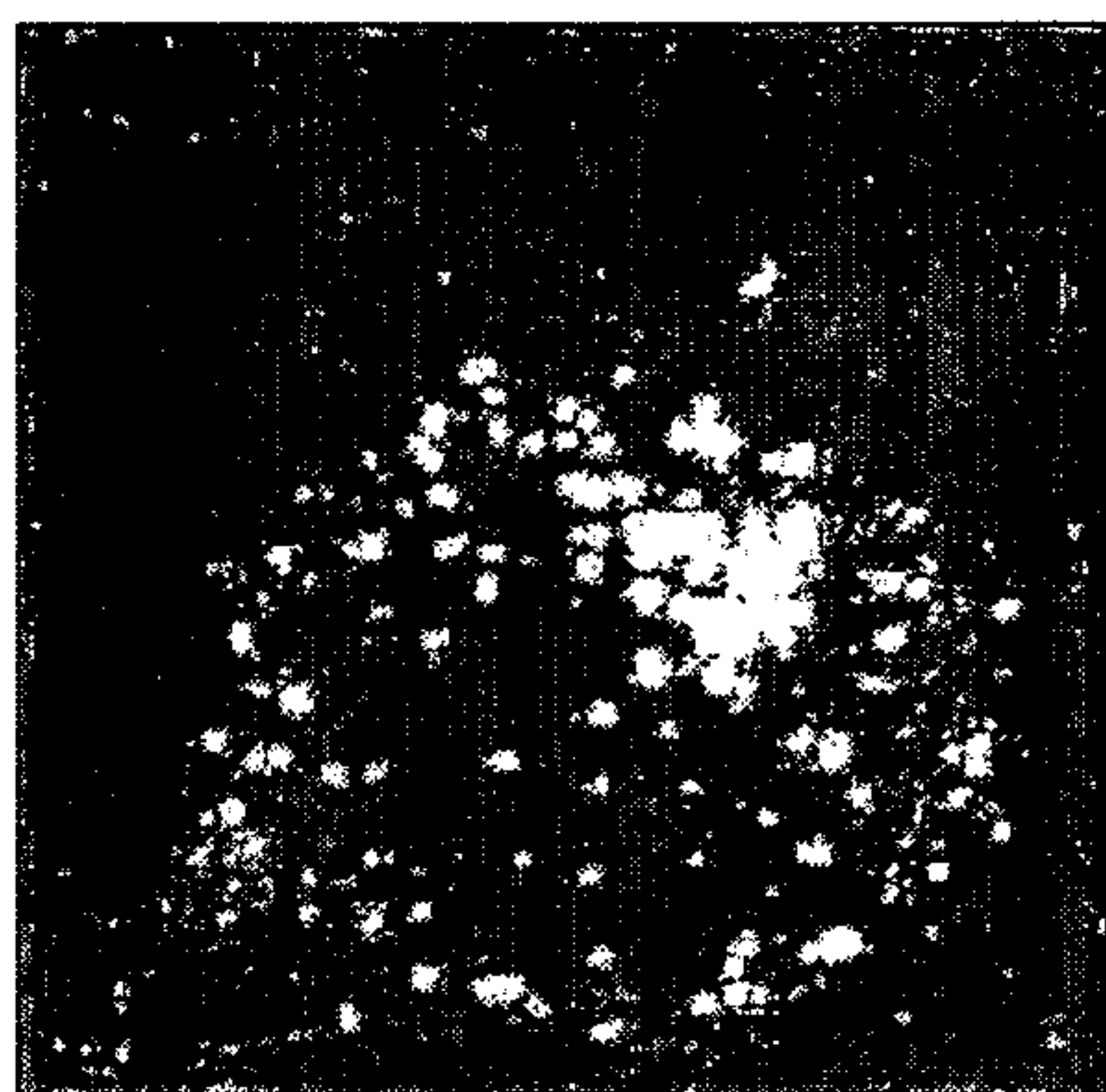
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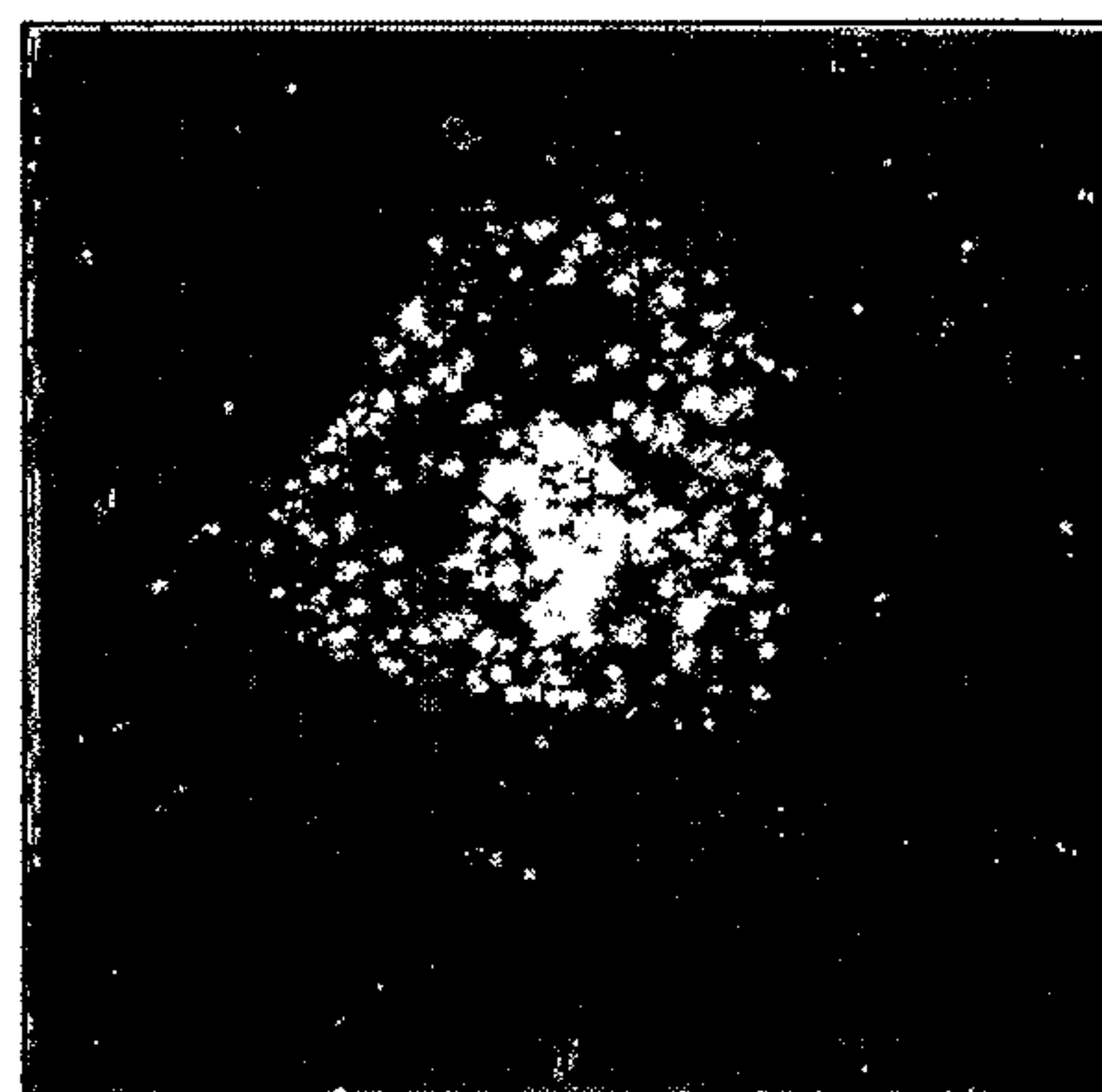
LN-GFP

FIG. 13G

LN-GFP

FIG. 13H

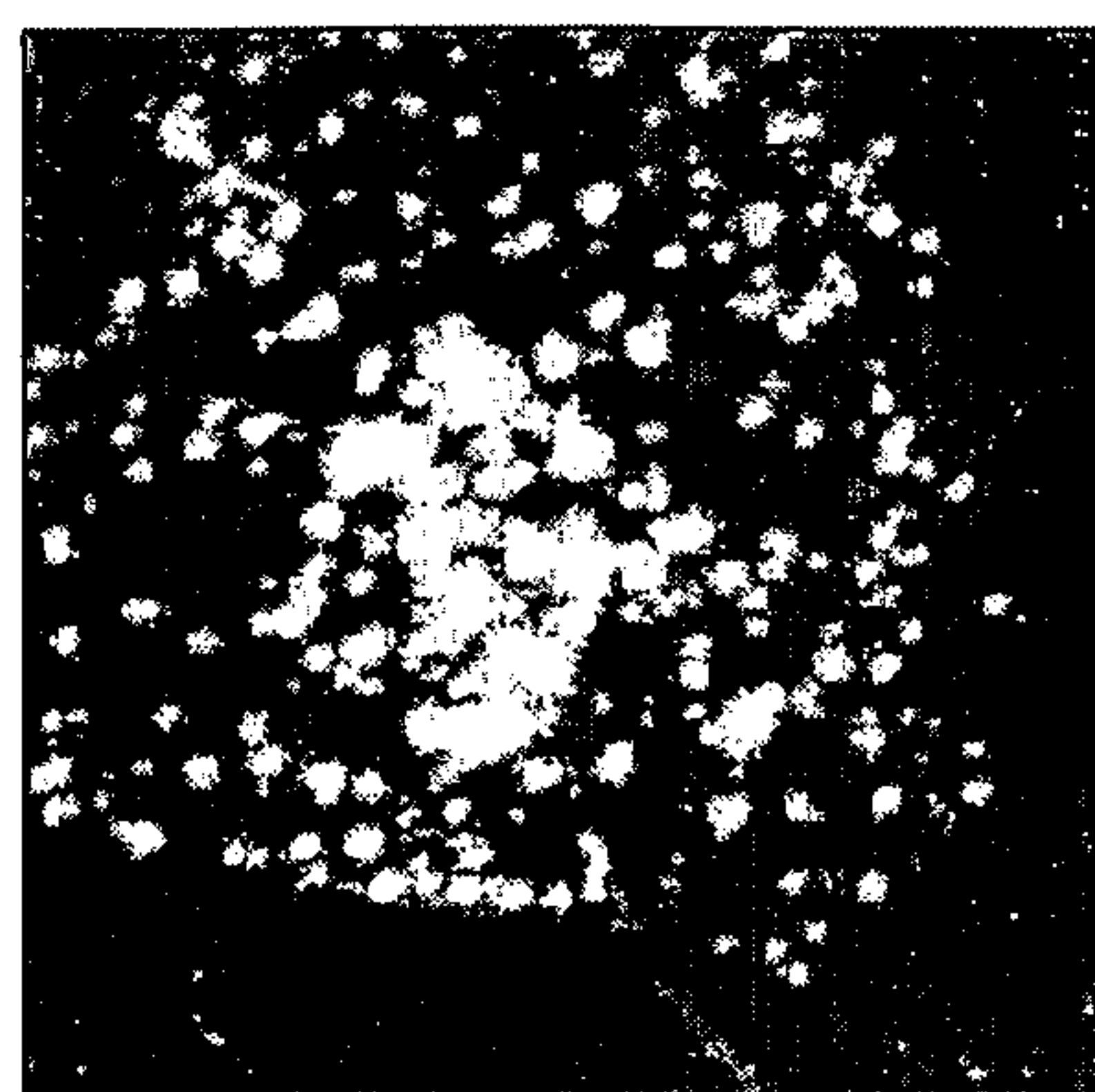
Merge

FIG. 13I

Merge

FIG. 13J

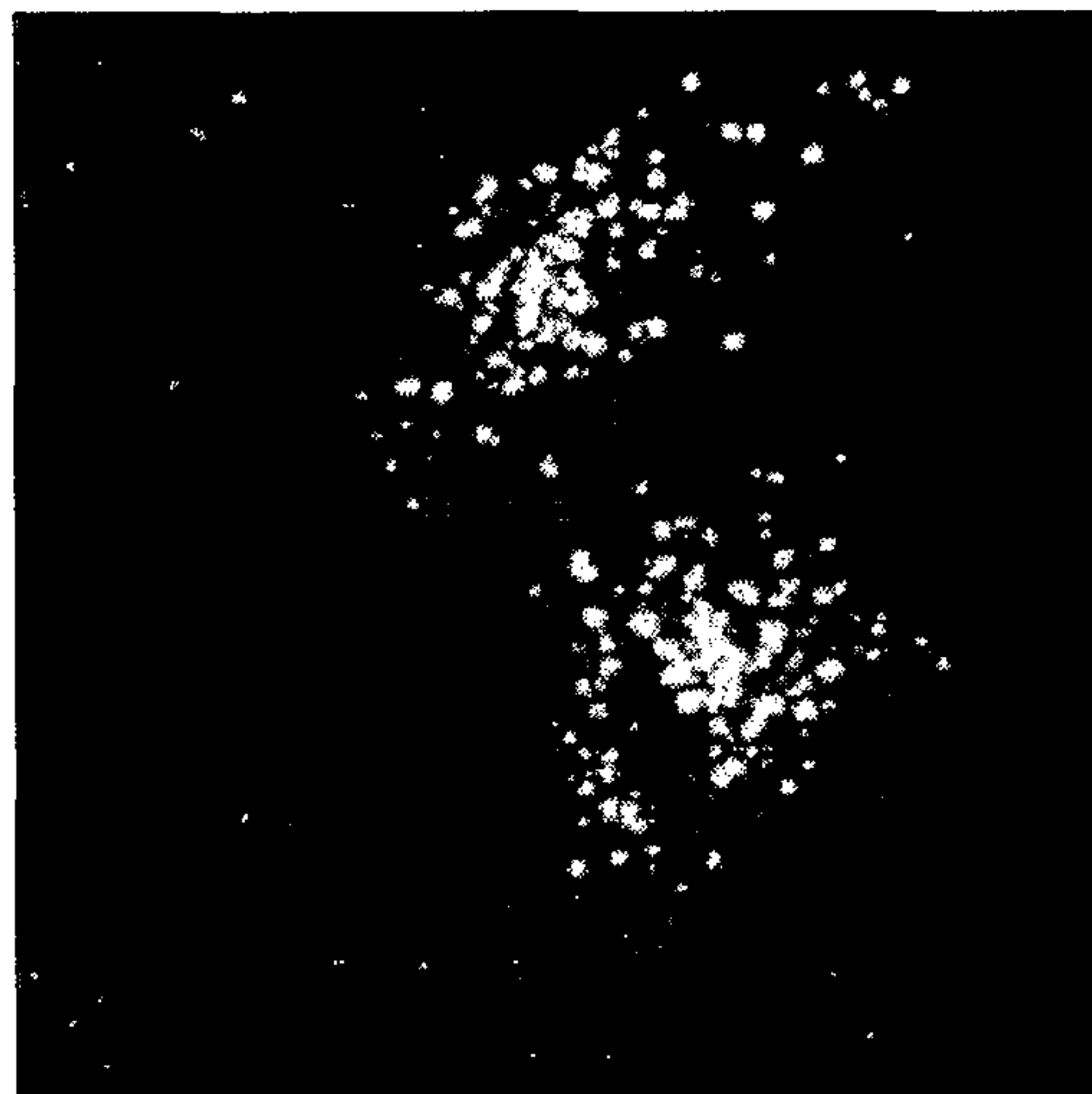
Merge

FIG. 13K

Merge

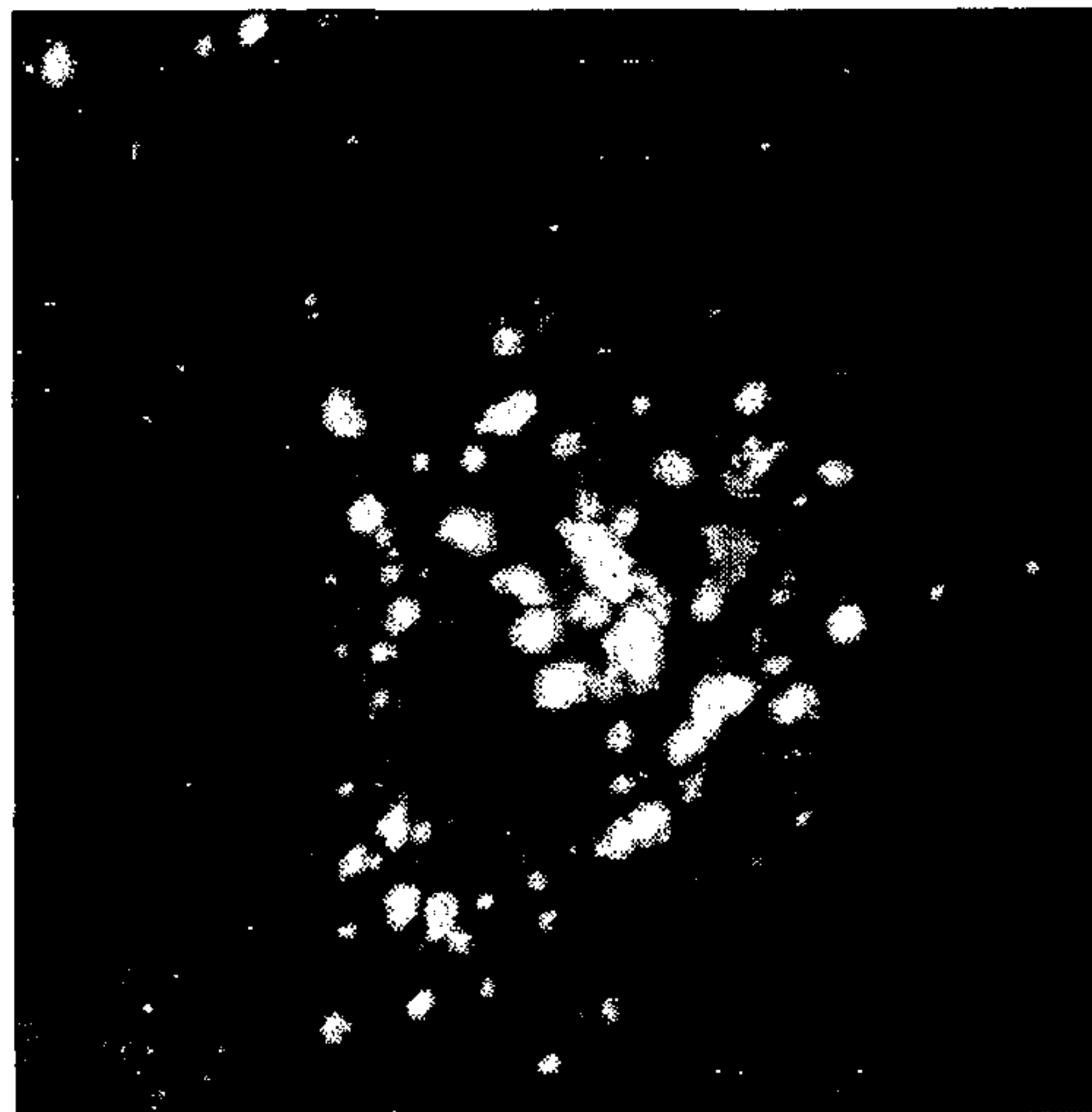
FIG. 13L

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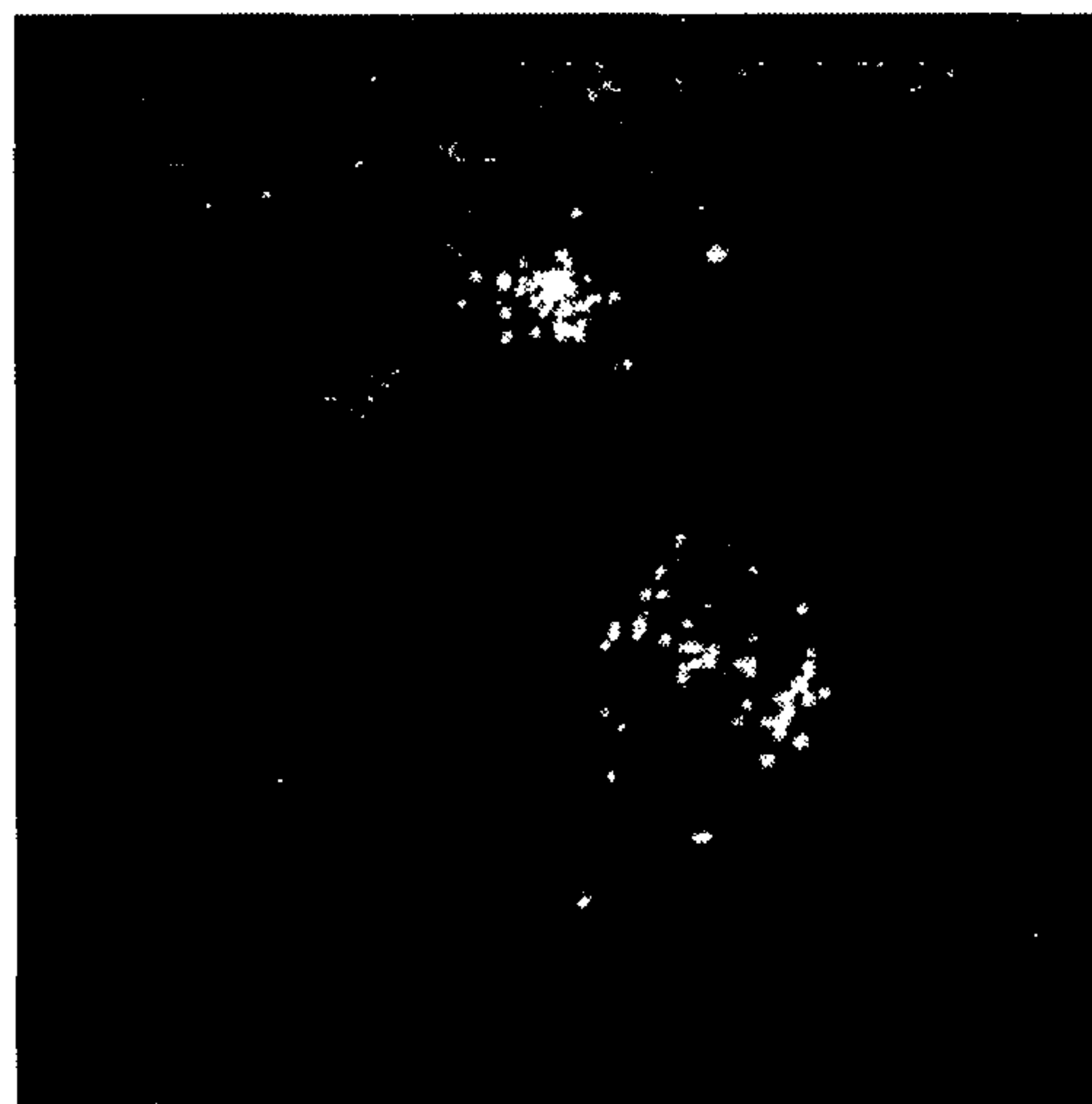
Golgi

FIG. 14A



Golgi

FIG. 14B



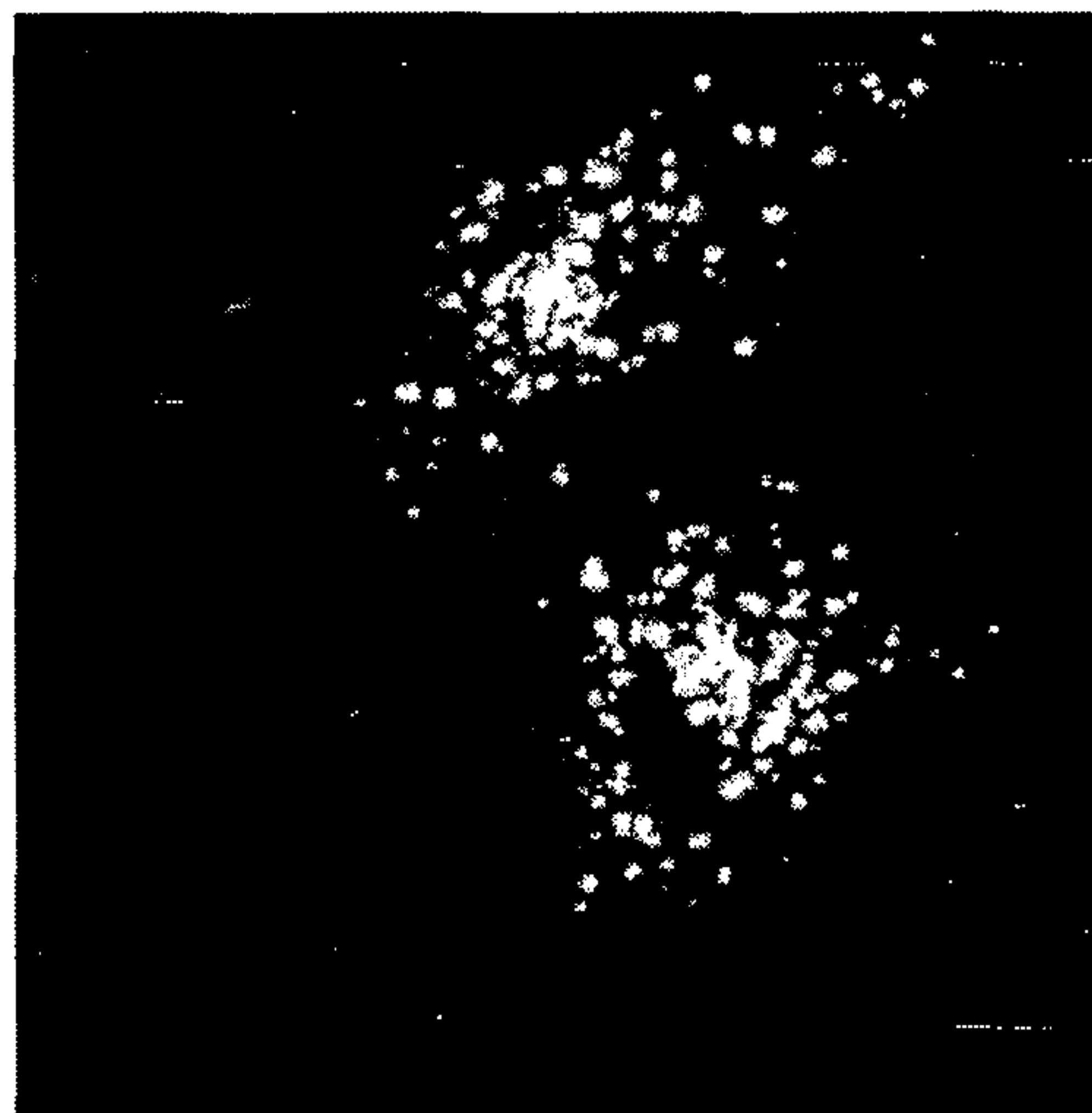
LN-GFP

FIG. 14C

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LN-GFP

FIG. 14D

Merge

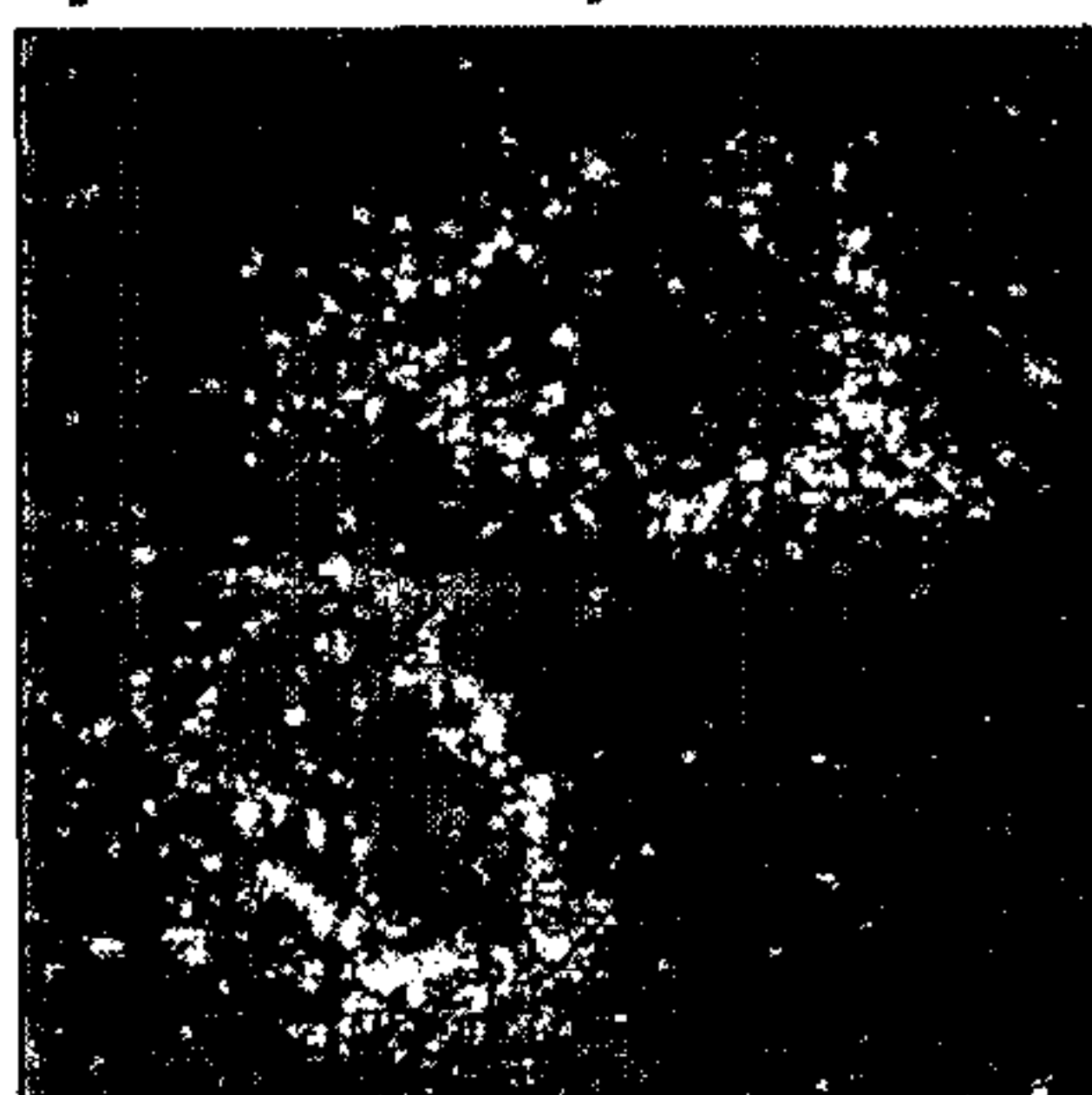
FIG. 14E

Merge

FIG. 14F

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HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



LNgfp

FIG. 15A

HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



LNgfp

FIG. 15B

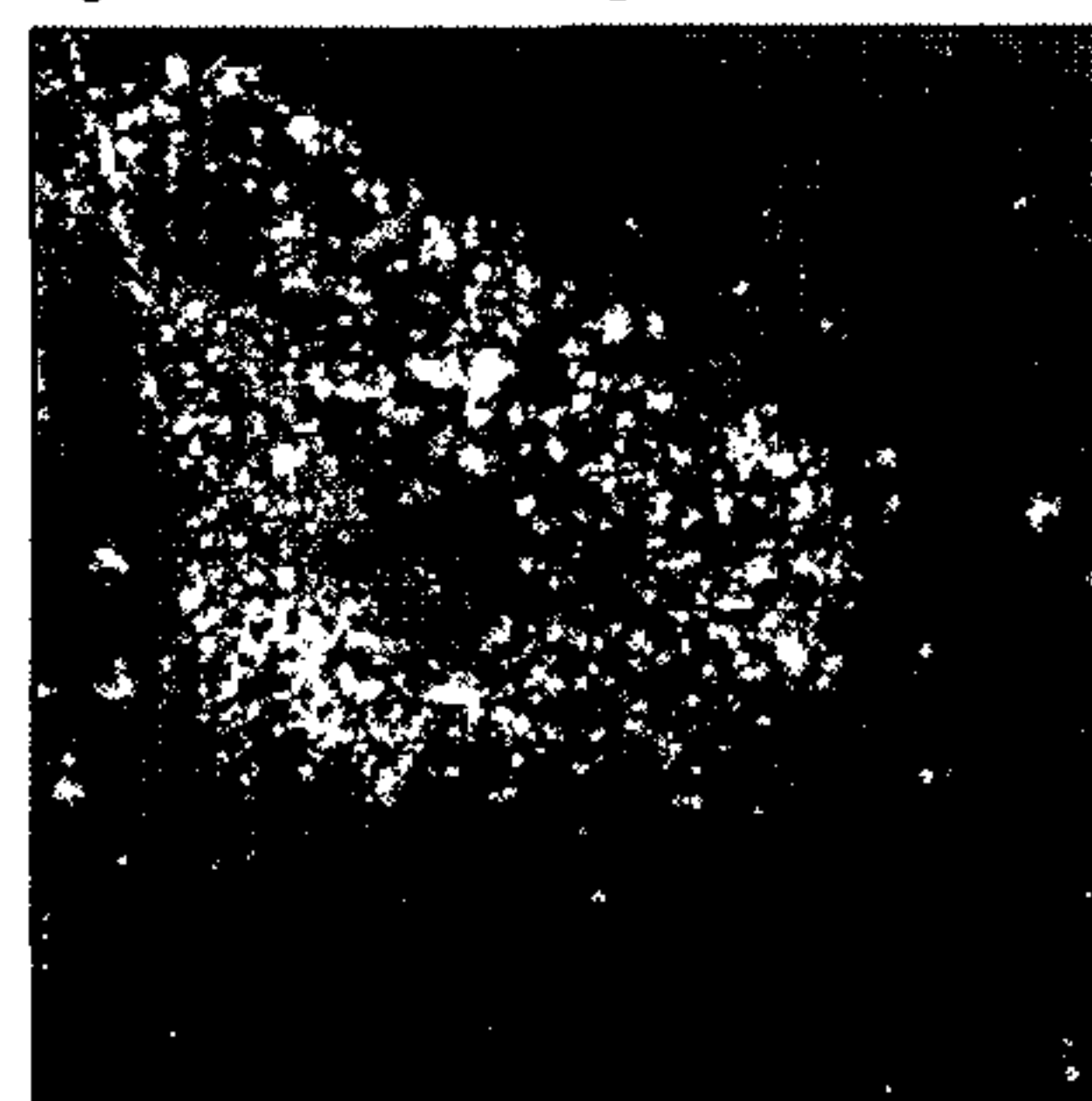
HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



LNgfp

FIG. 15C

HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



LNgfp

FIG. 15D

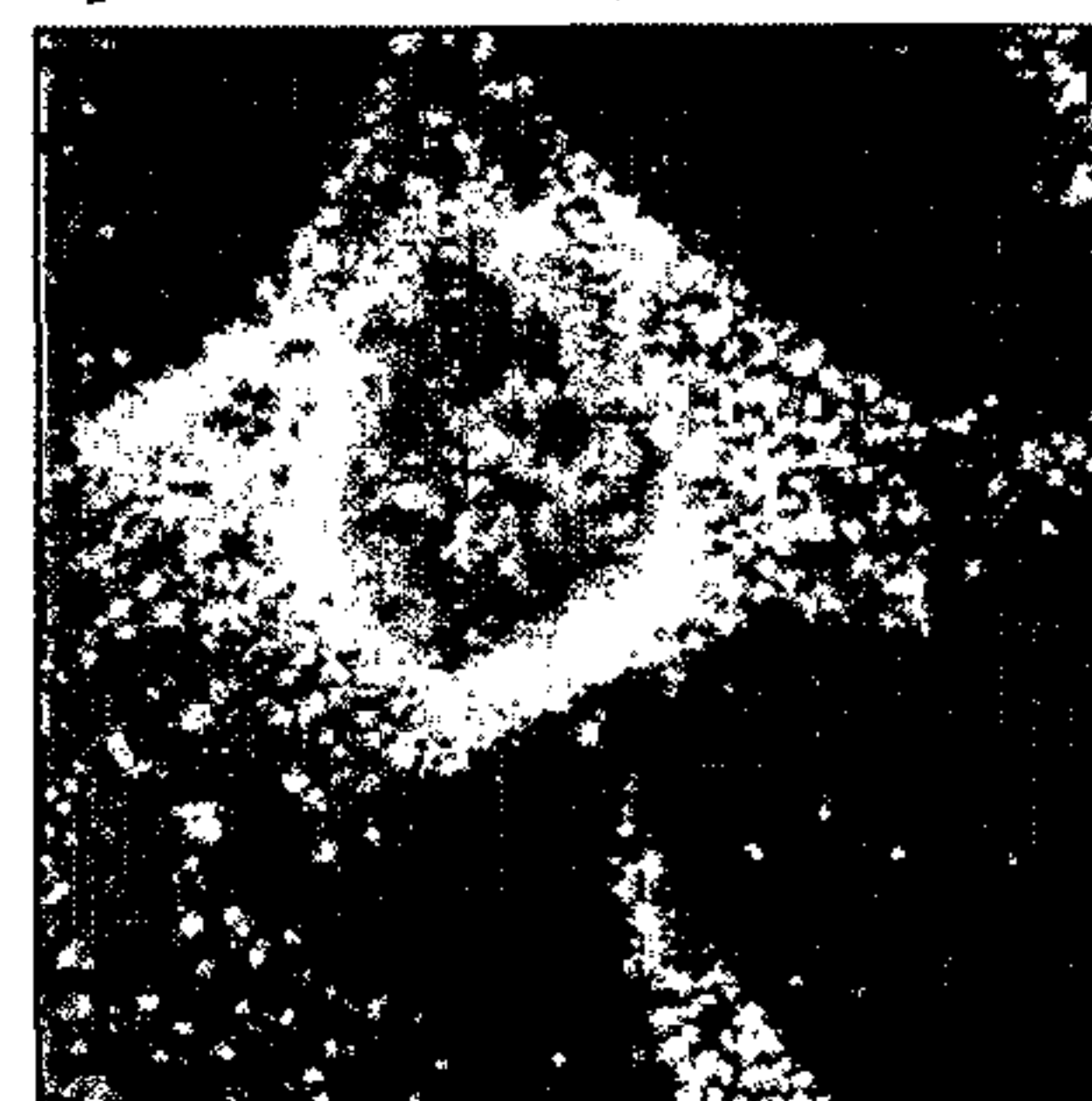
HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



20S

FIG. 15E

HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



20S

FIG. 15F

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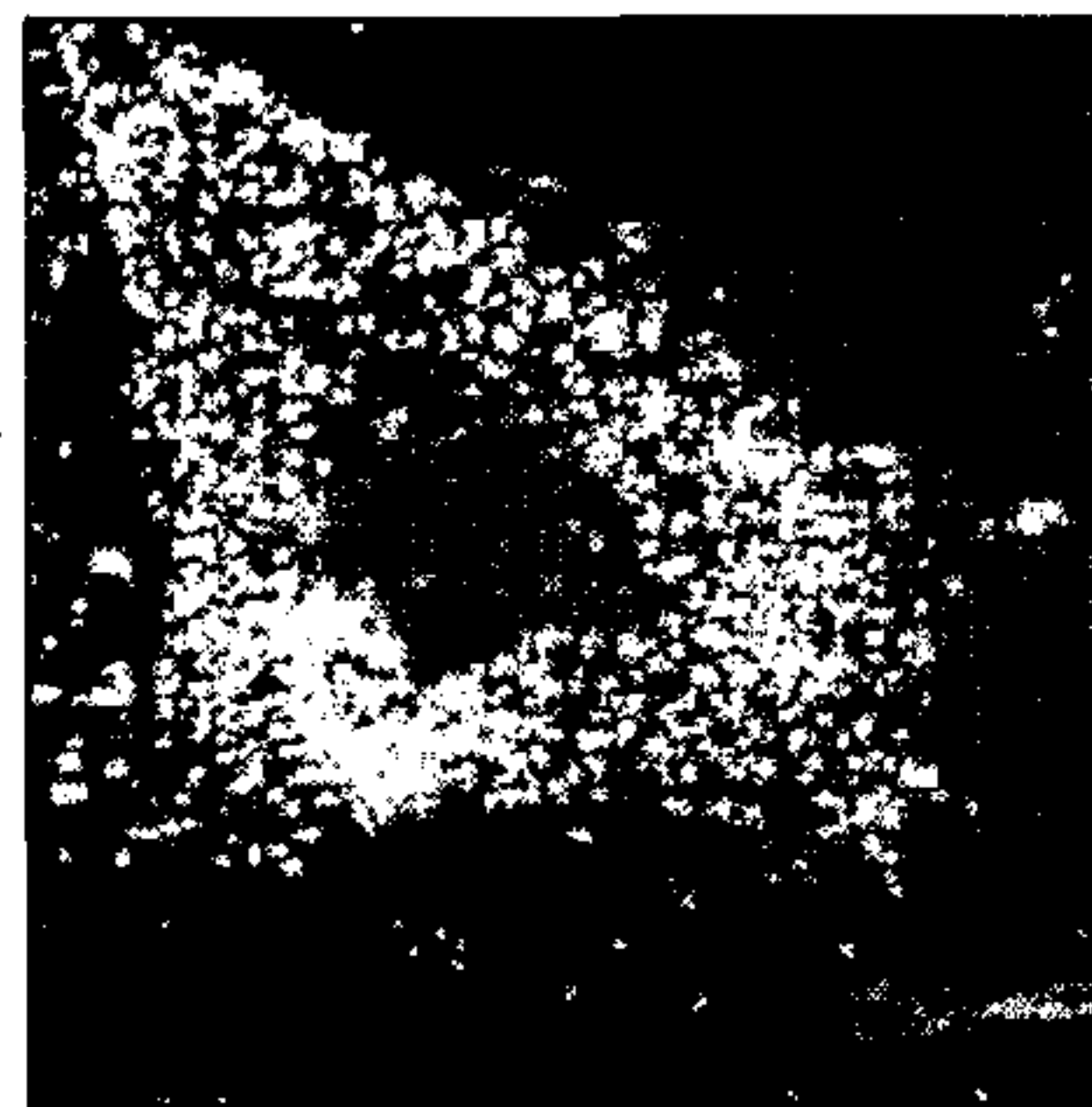
HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



20S

FIG. 15G

HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



20S

FIG. 15H

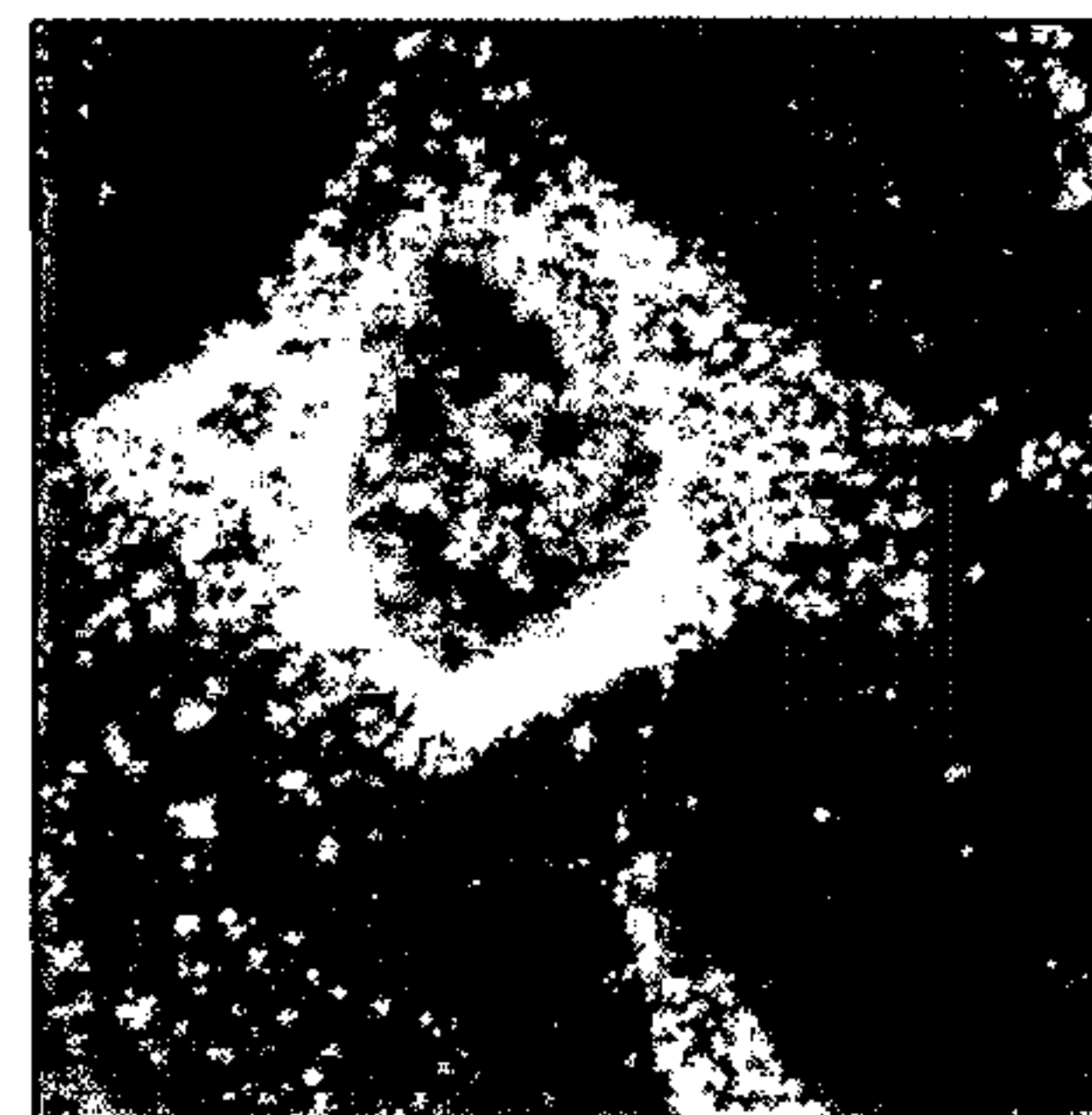
HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



Merge

FIG. 15I

HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



Merge

FIG. 15J

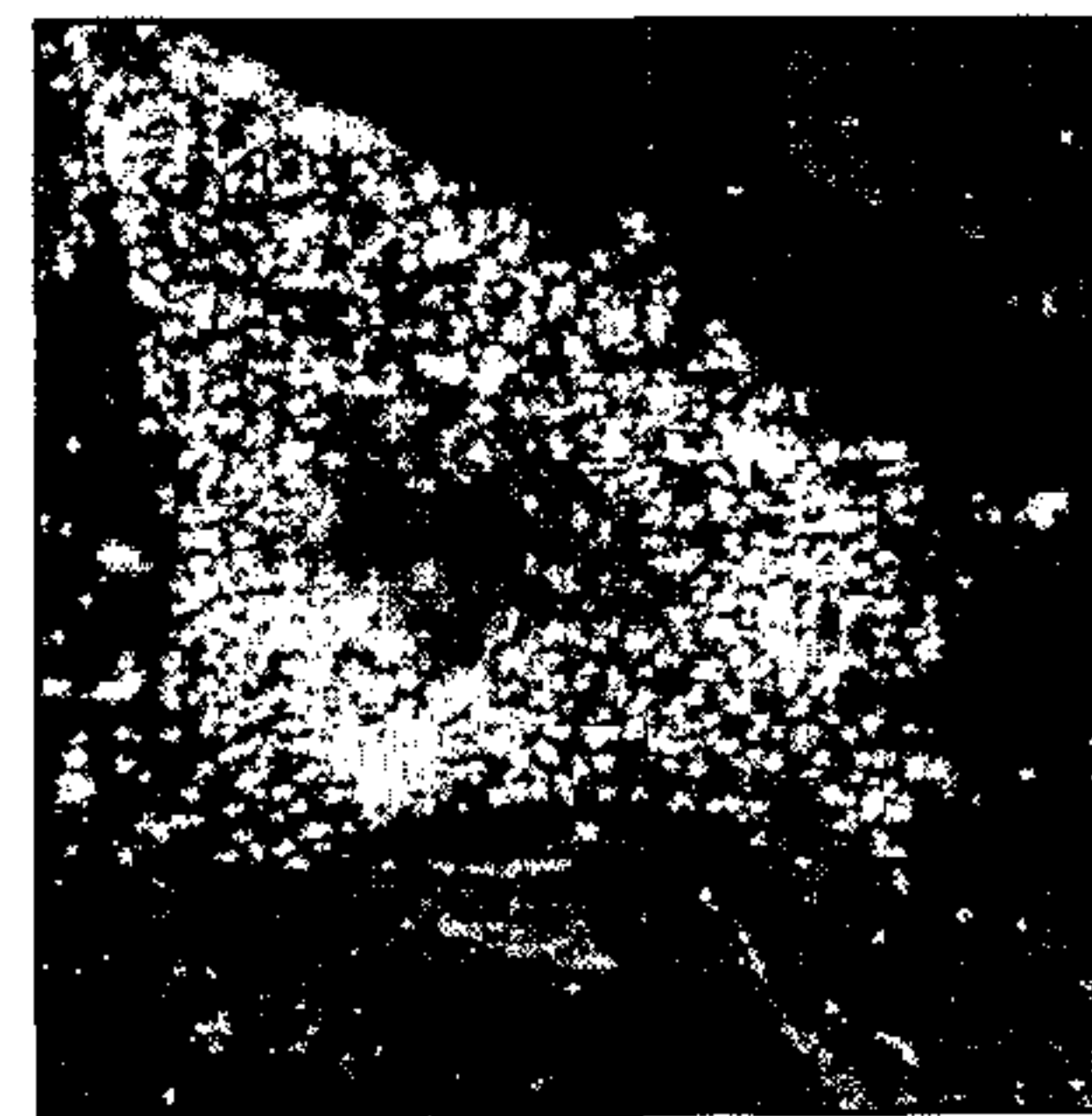
HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



Merge

FIG. 15K

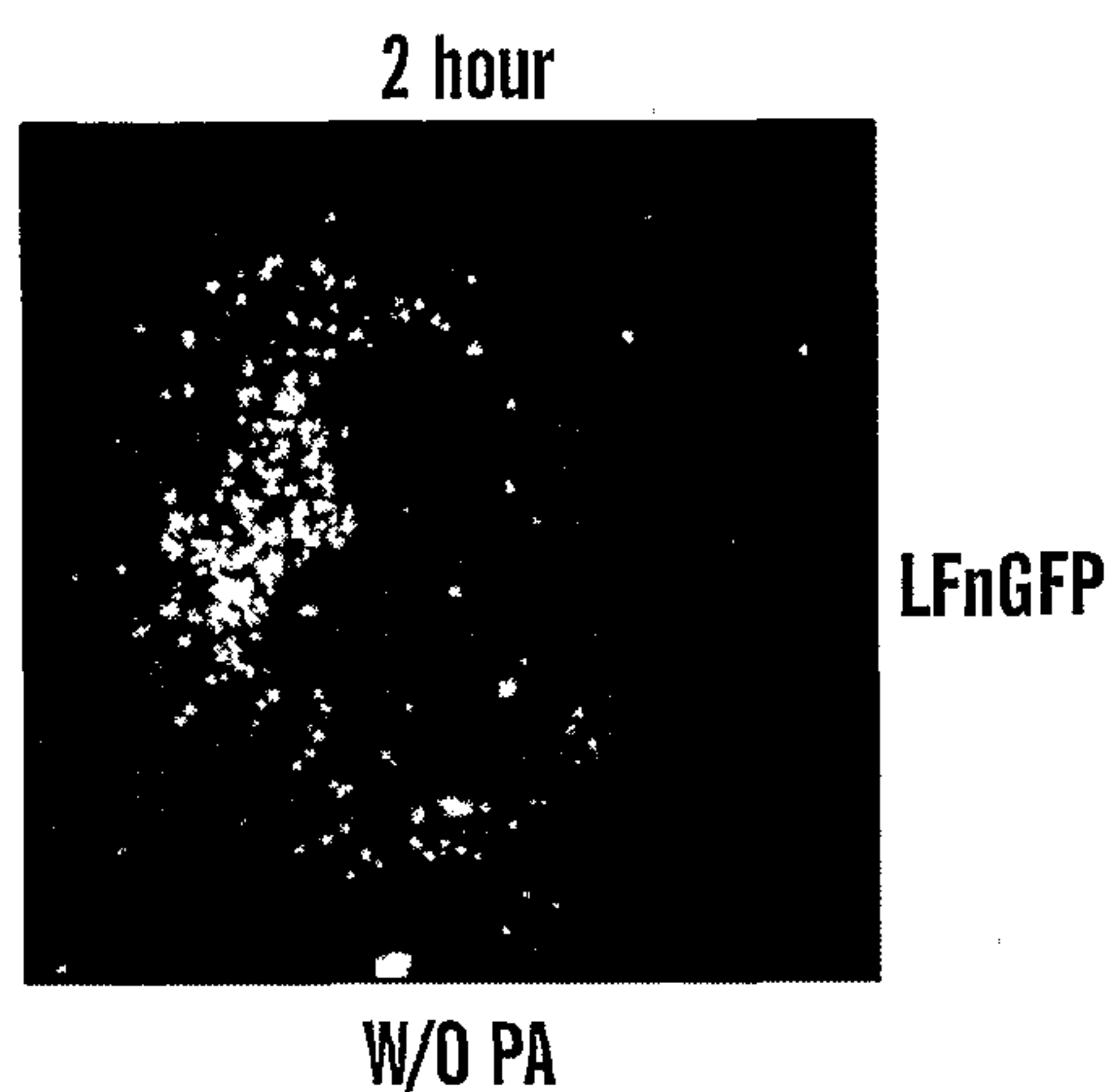
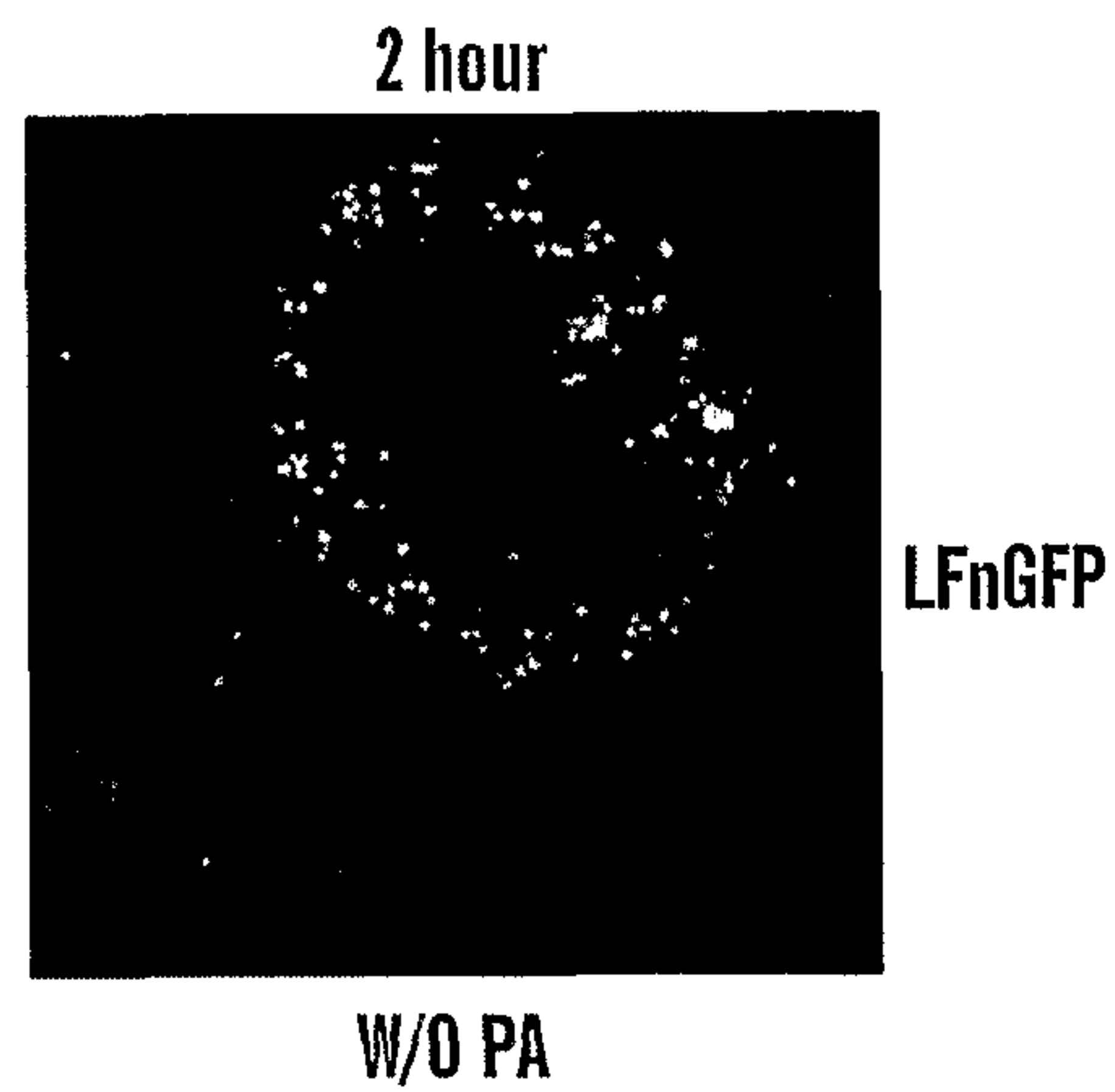
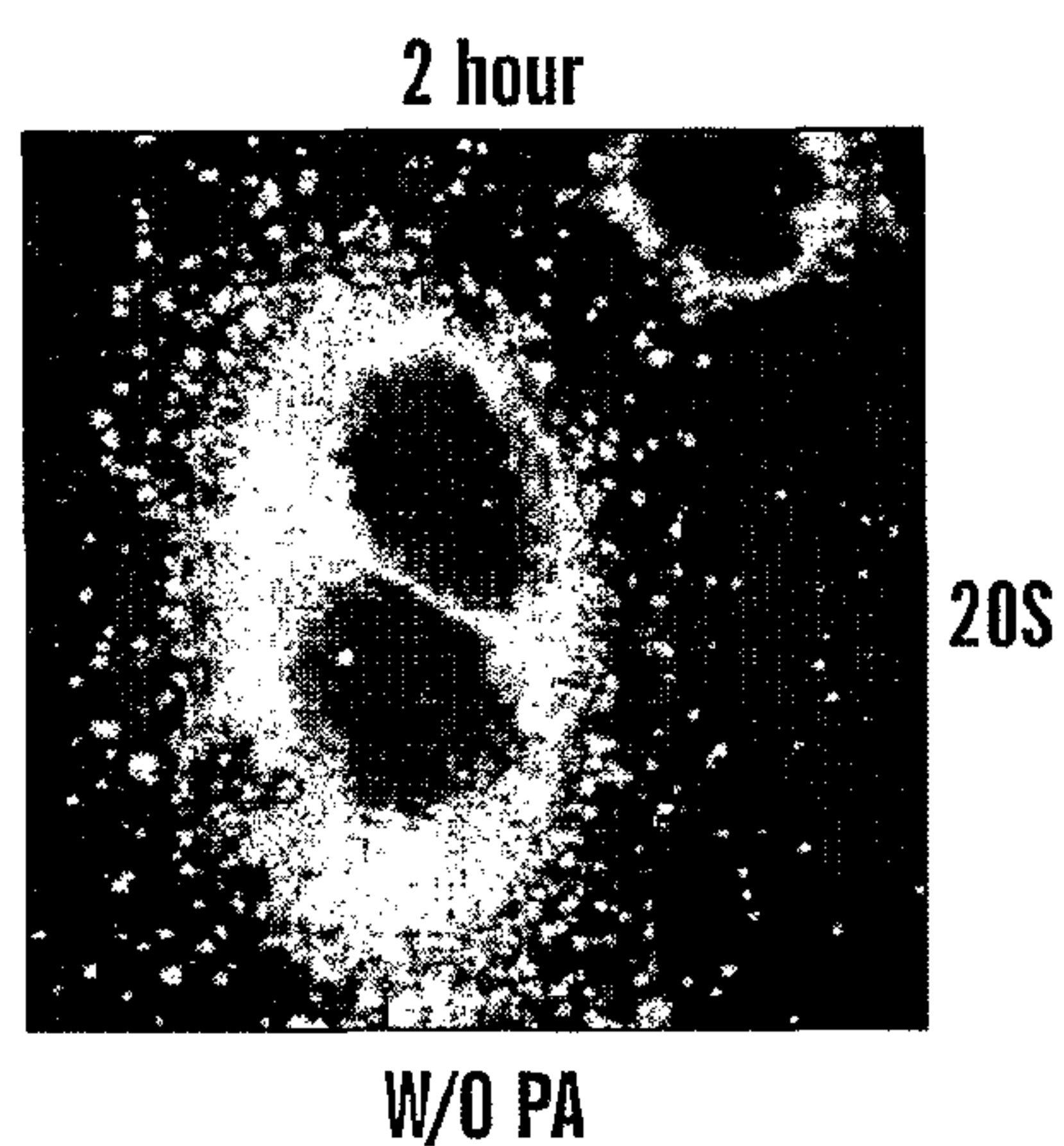
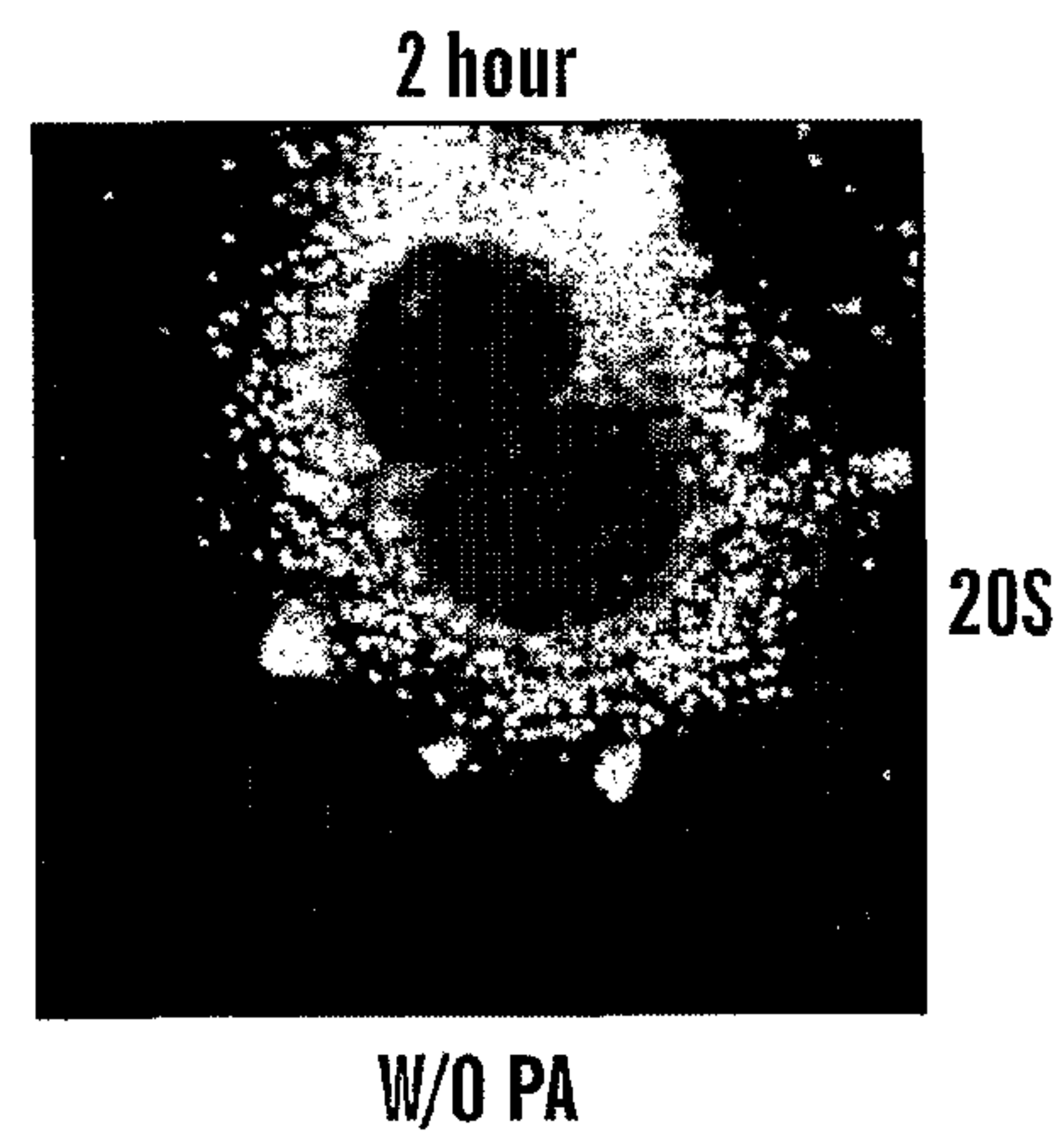
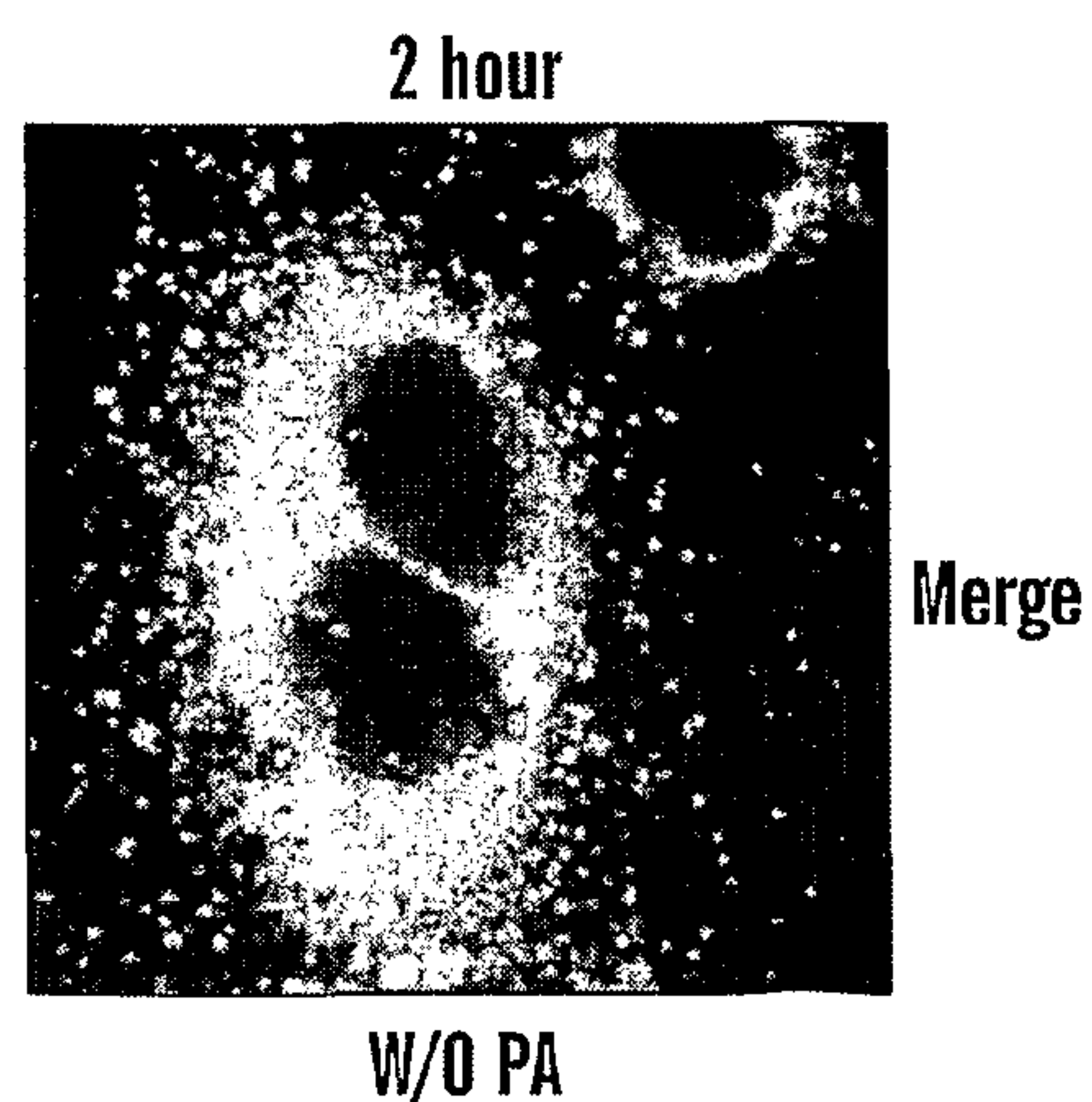
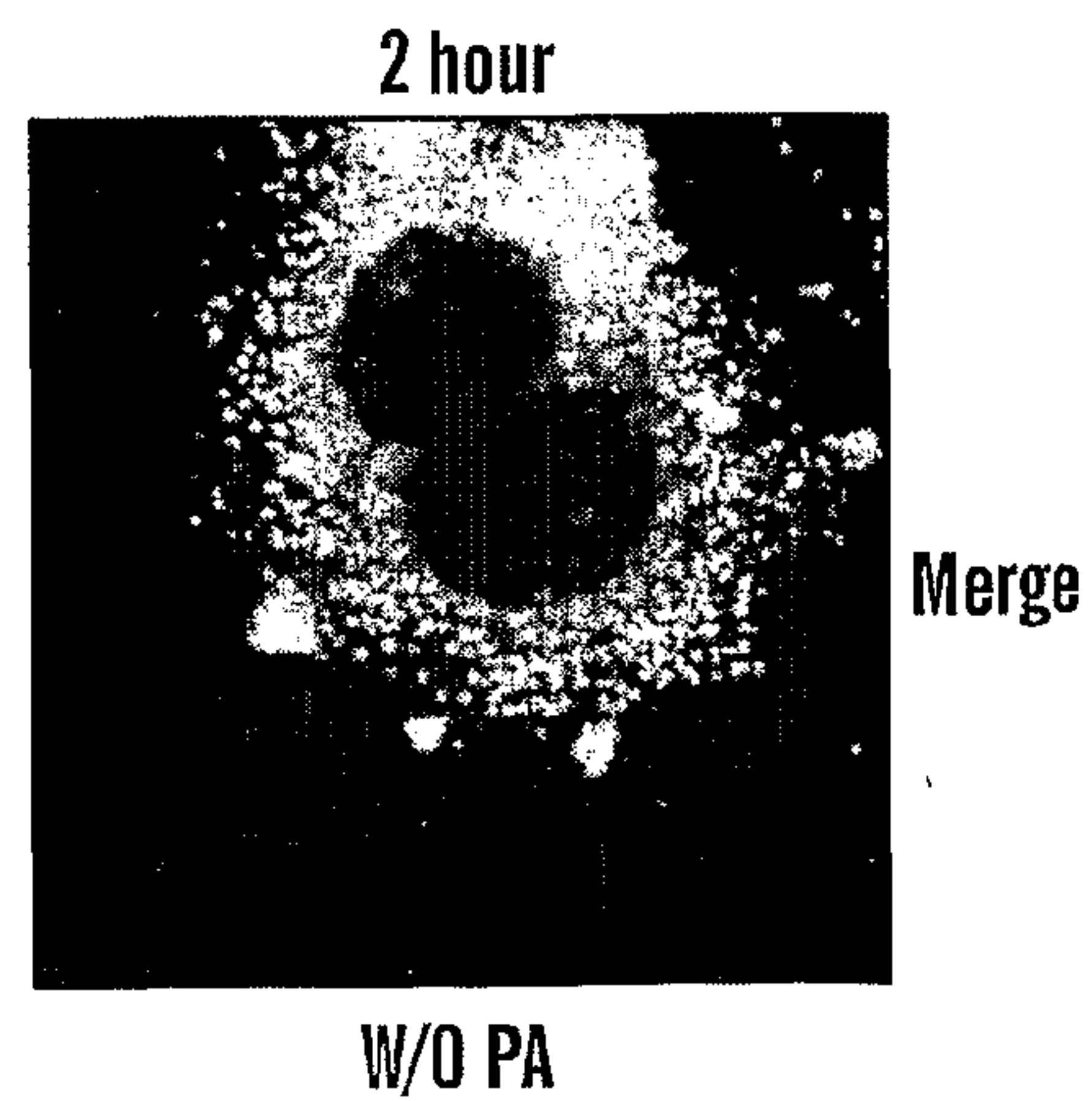
HeLa cells were incubated with 40 $\mu\text{g/ml}$ LNgfp for 2h @37 C, then fixed and stained with anti-20s preteasome polyclonal antibody (CalBiochem).



Merge

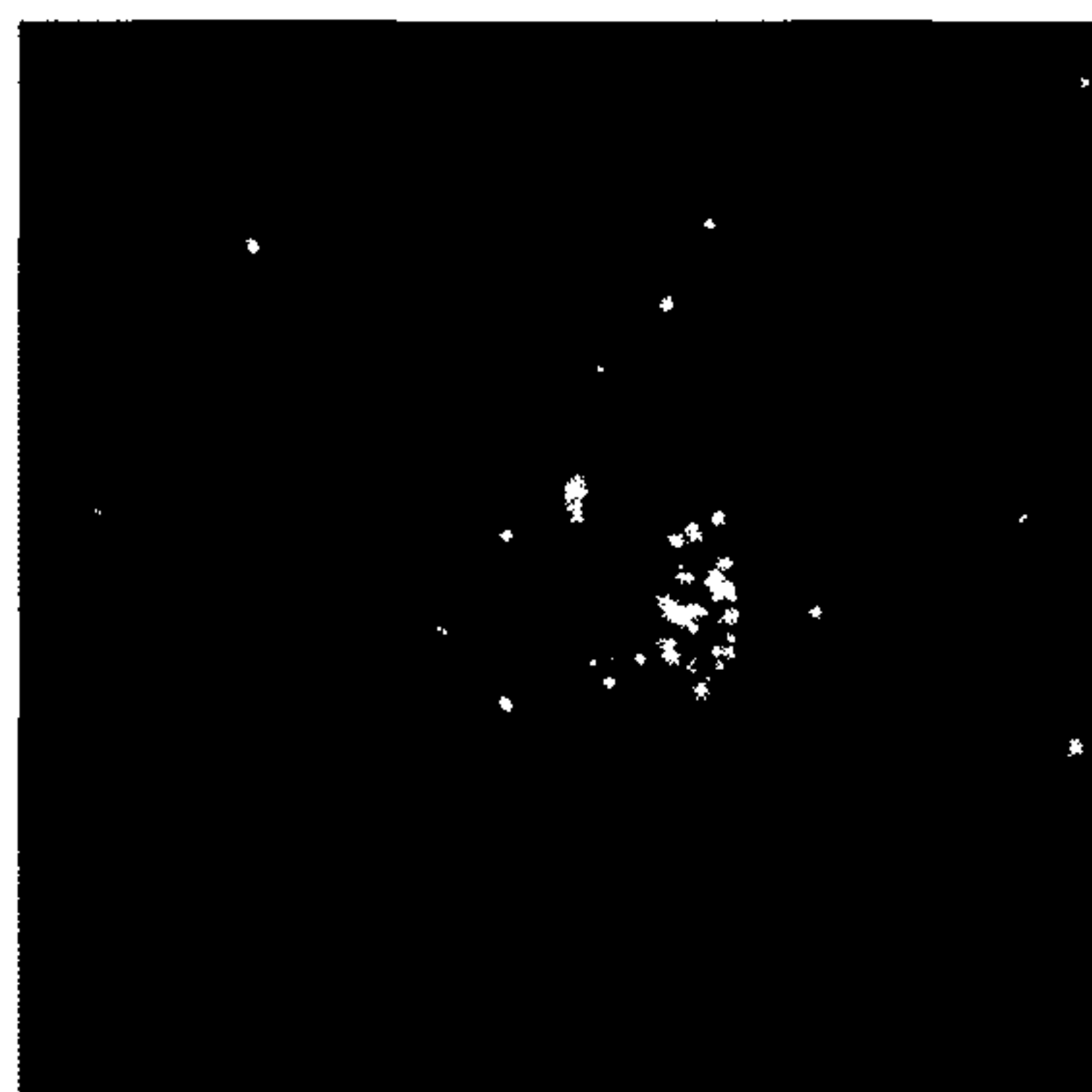
FIG. 15L

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**FIG. 16A****FIG. 16B****FIG. 16C****FIG. 16D****FIG. 16E****FIG. 16F**

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2 hour

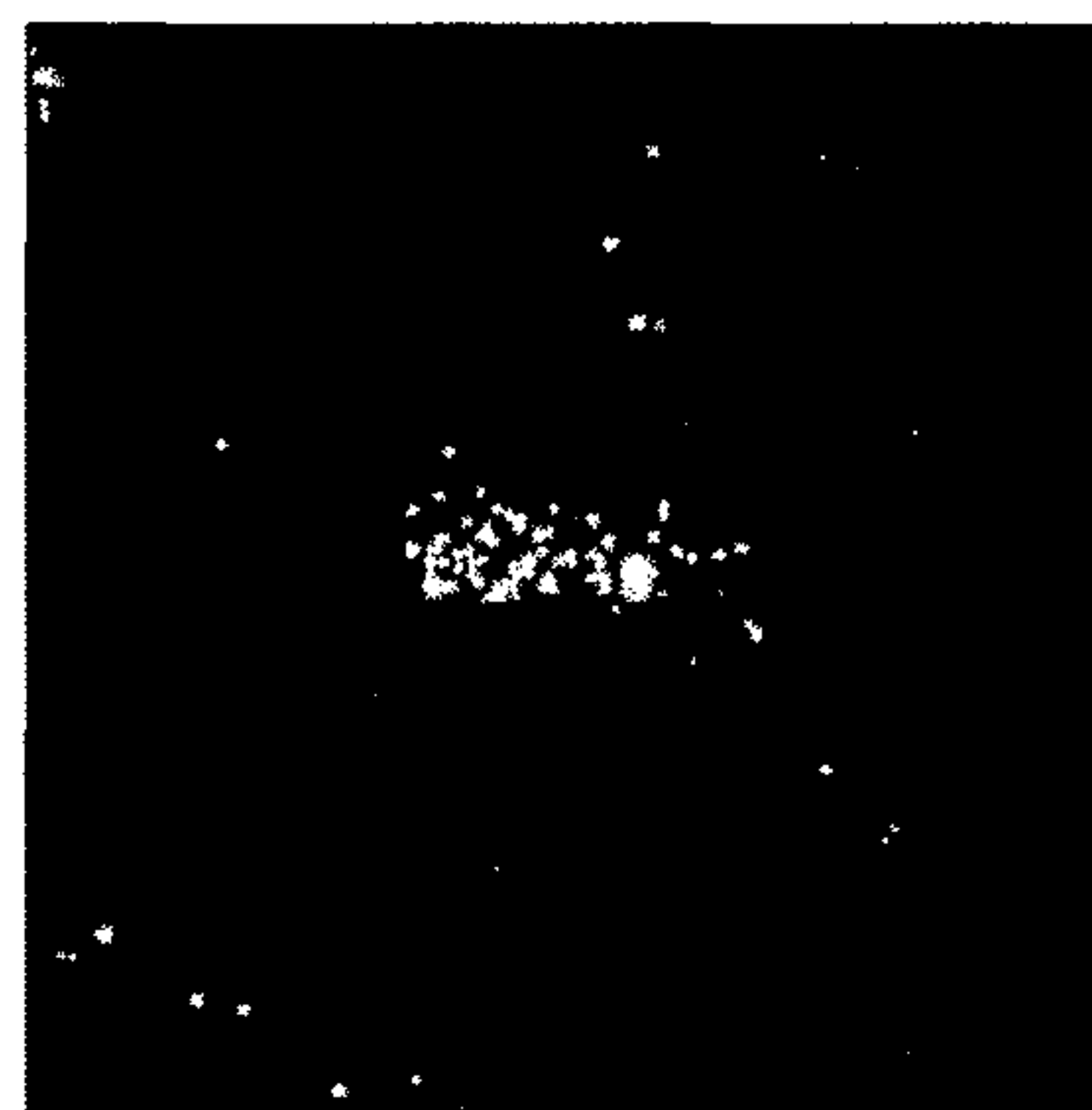


LFnGFP

With PA

FIG. 16G

2 hour

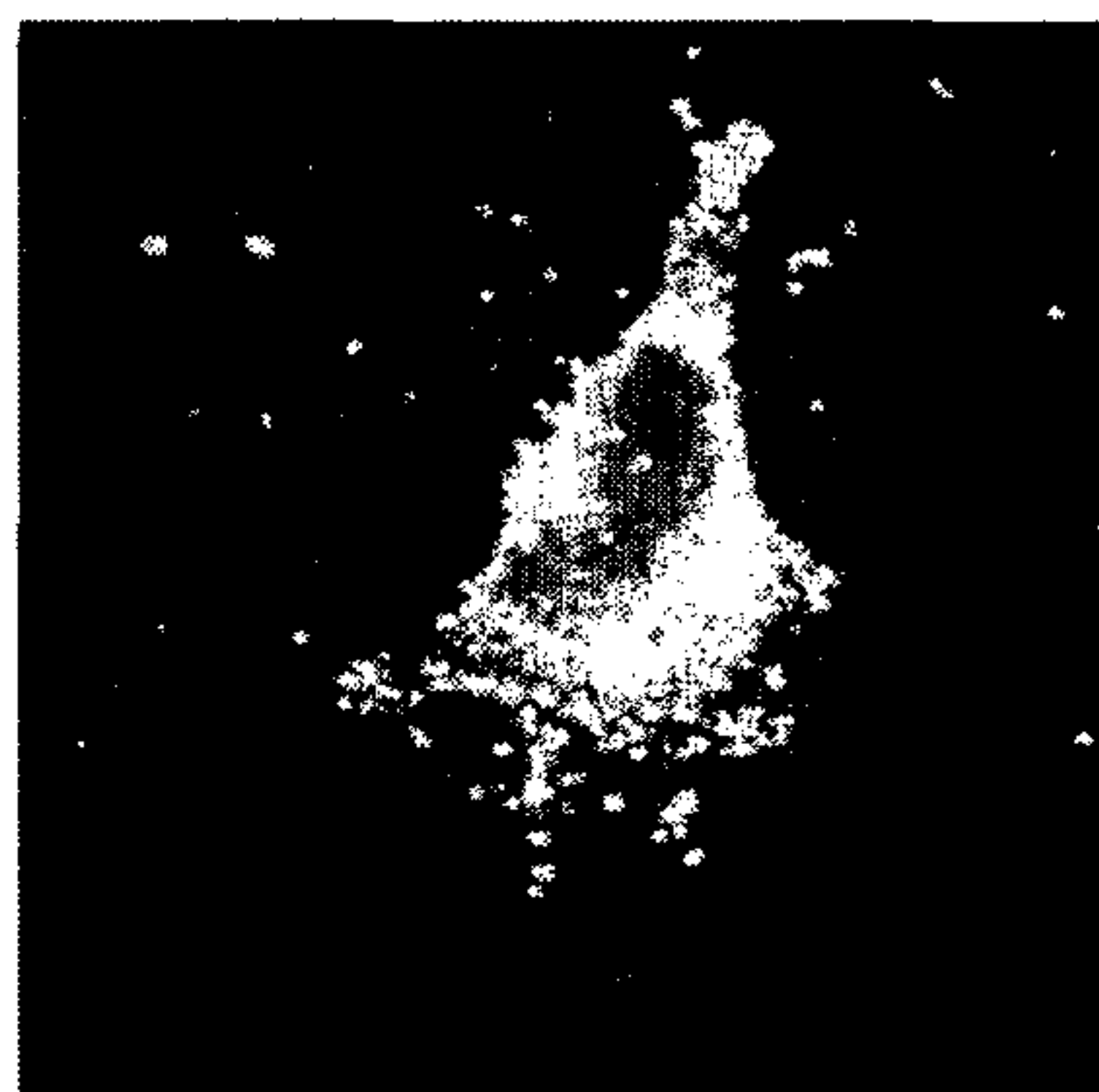


LFnGFP

With PA

FIG. 16H

2 hour

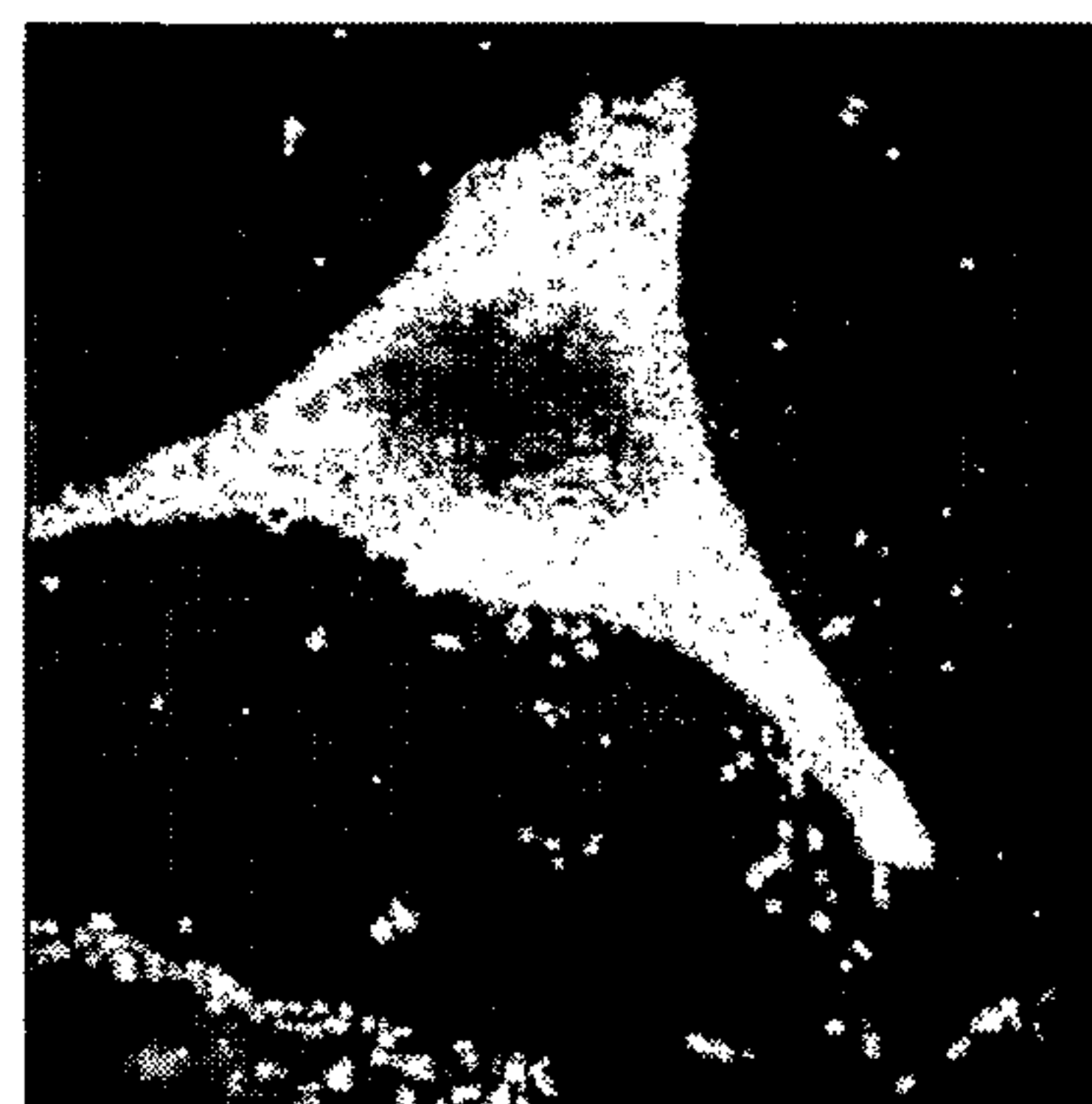


20S

With PA

FIG. 16I

2 hour



20S

With PA

FIG. 16J

2 hour

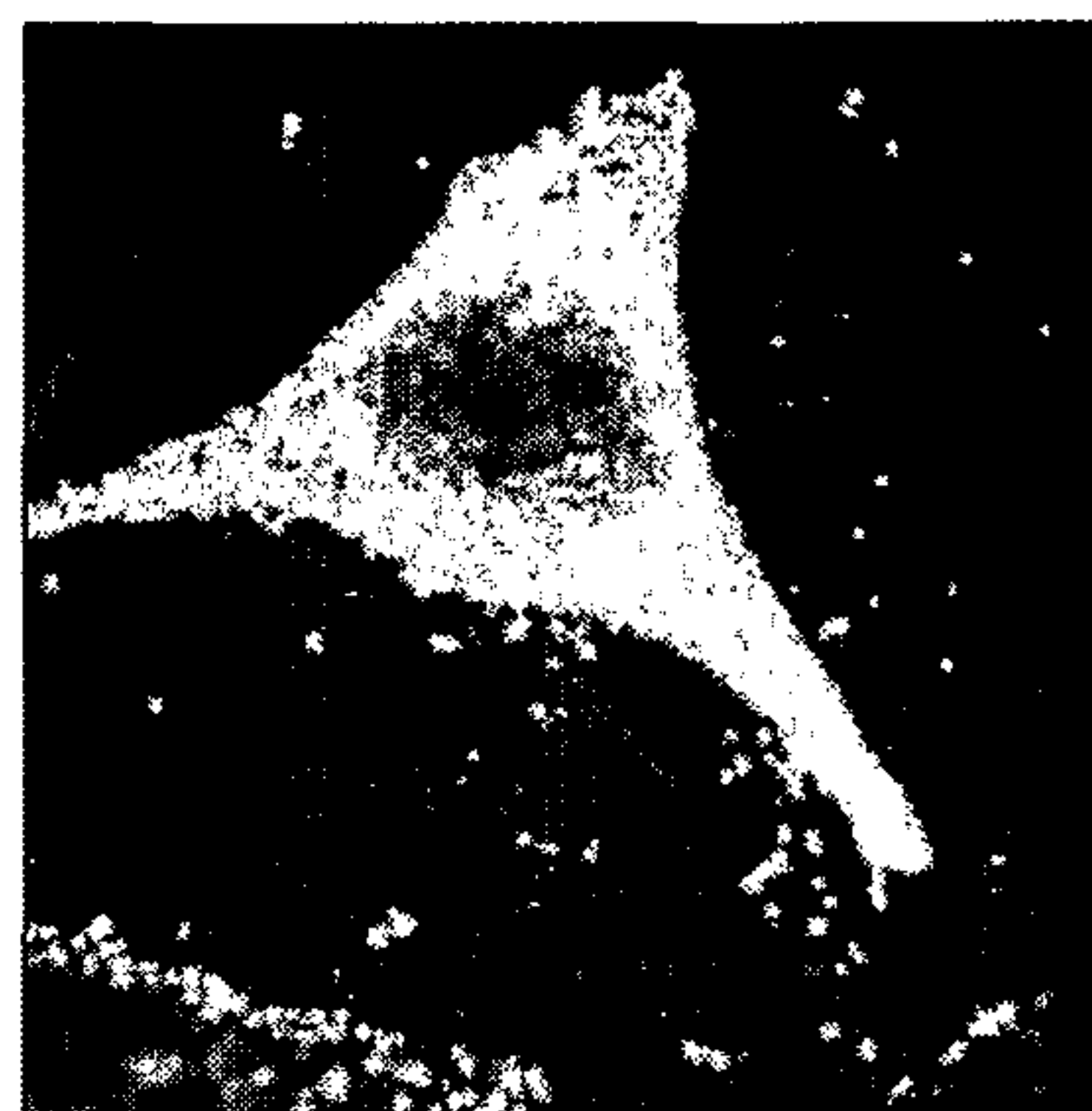


Merge

With PA

FIG. 16K

2 hour

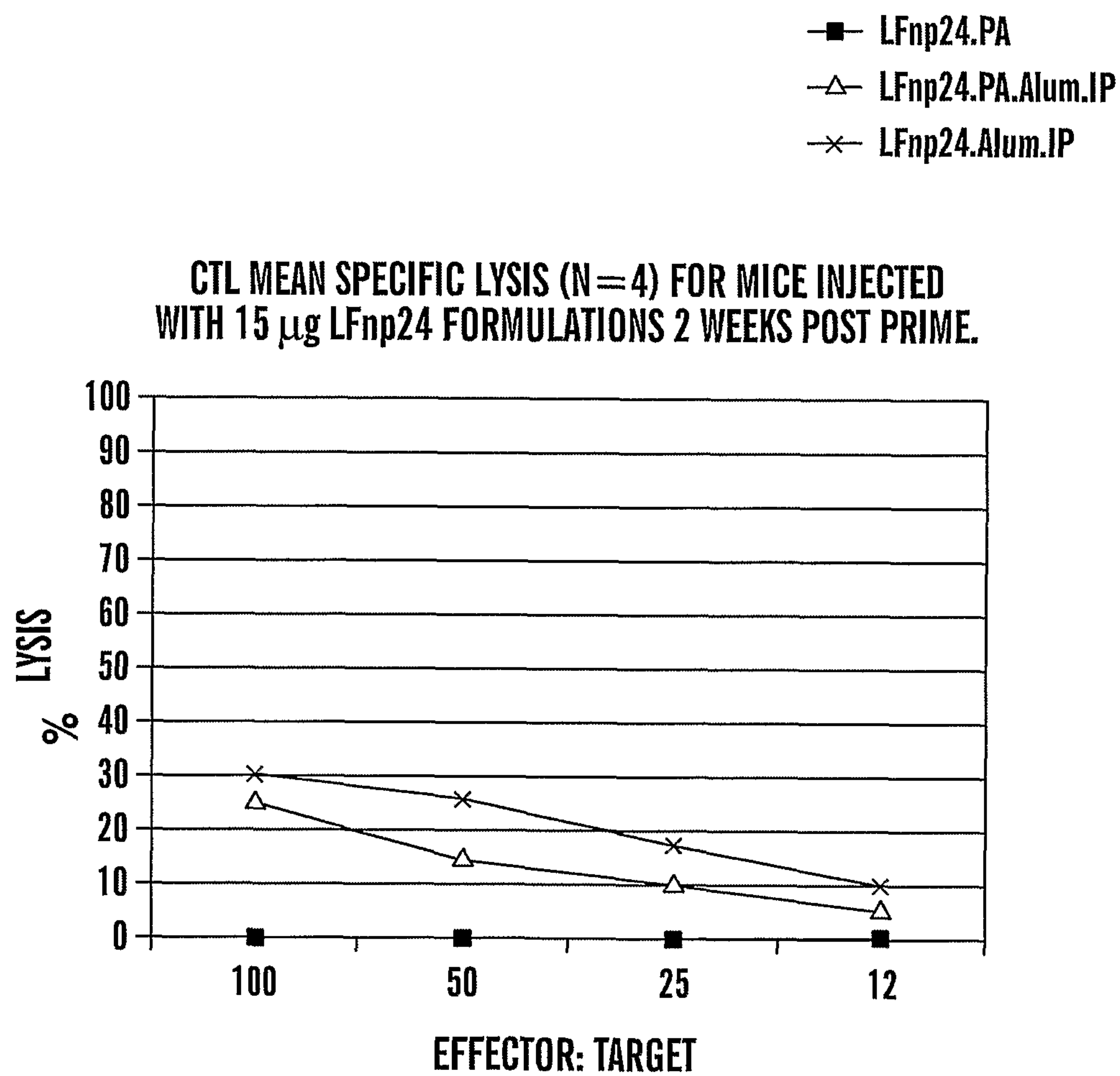


Merge

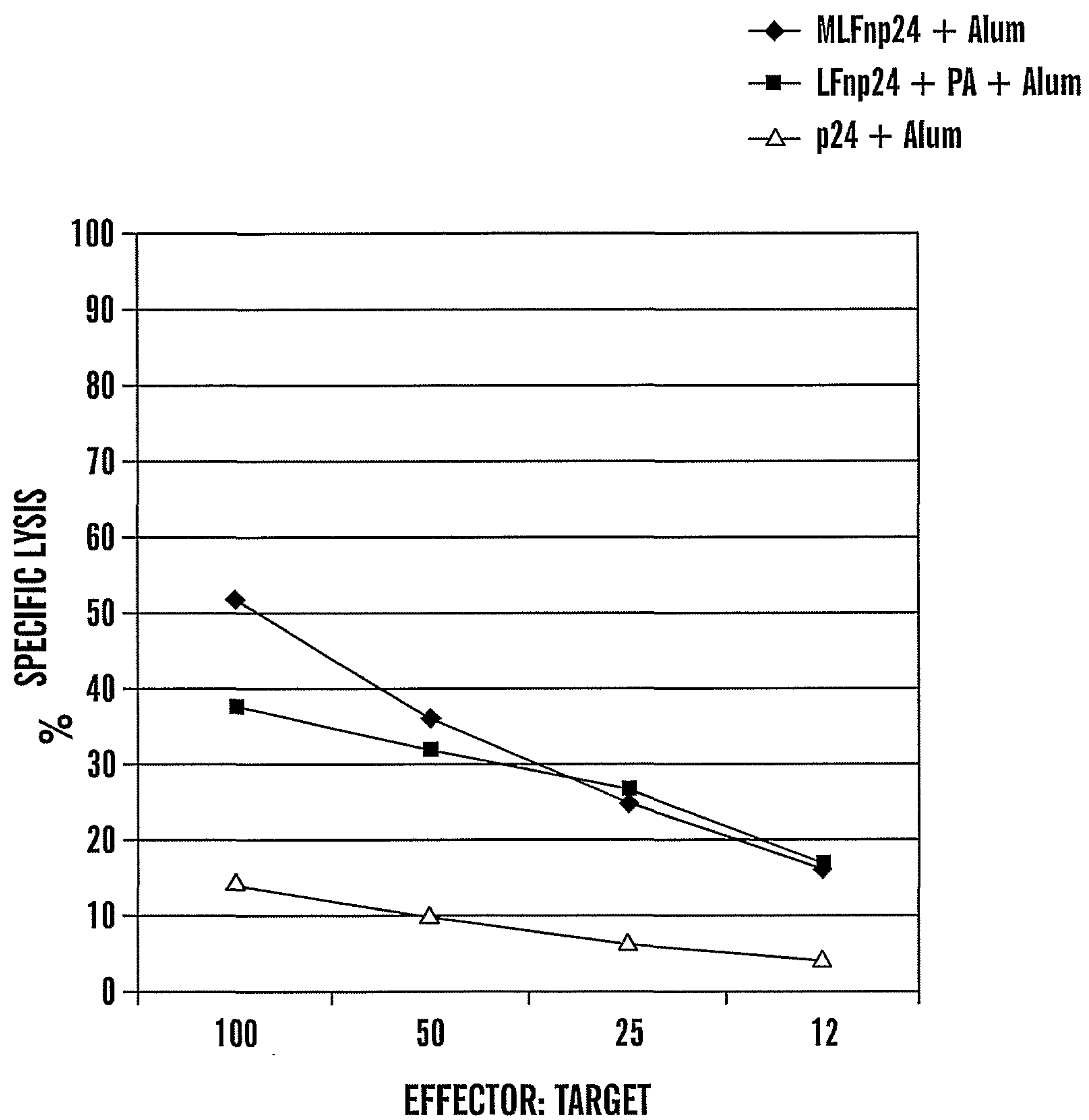
With PA

FIG. 16L

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**FIG. 17**

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**FIG. 18**