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Accumulation of radiolabelled low molecular peptides and proteins in experimental inflammation

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Abstract

This study focuses on evaluating accumulation of the low molecular peptides and proteins labelled with ^{99m}Tc in rat inflammatory/infection foci. Peptides (human leukocyte dialysate, HLD; thymosin fraction 5, TF5; aprotinin, APT), and proteins (human IgG, HIG) were labelled with ^{99m}Tc using redox polymer. The labelling efficiency was evaluated using paper, TLC and/or column chromatography. Biodistribution of labelled substances was evaluated in rats with *Staphylococcus aureus* infection or with sterile kaolin suspension inflammation 24 h after abscess induction. Accumulation of ^{99m}Tc activity was determinated both by external gamma camera imaging and by counting dissected tissues 4 h after administration. The evaluated peptides and proteins show high labelling efficiency (^{99m}Tc-HLD > 98%, ^{99m}Tc-TF5 > 95%, ^{99m}Tc-APT > 98%, ^{99m}Tc-HIG > 95%). Usage of redox polymer for labelling increases the stability of ^{99m}Tc-labelled substances. The labelling efficiency stays nearly the same (95–98%) after 8 h at least. In experimentally induced inflammation the amount of ^{99m}Tc-peptides and ^{99m}Tc-HIG activity accumulated is 2.5–6.5 and 5.3–10.6 times higher than in a control tissue. When comparing two types of model inflammations (kaolin- and *Staphylococcus*-induced ones), the values measured with ^{99m}Tc-peptides are more than double than those of kaolin suspension inflammation. The studied low molecular peptides labelled with ^{99m}Tc allow rapid localisation of infection foci. ^{99m}Tc labelled HIG proved useful for detection of infections and inflammatory lesions.

Keywords: Technetium (^{99m}Tc); Inflammation; Biodistribution; Peptides; Proteins

1. Introduction

Infectious diseases remain prominent in clinical medicine. All inflammatory processes, infectious and

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non-infectious, develop along a known sequence-leakage of fluid, followed by consequent leakage of small molecules, proteins, then by diapedesis (transudation) of cells, and local accumulation of cells. From what is known about inflammation we can predict which radiopharmaceutical might have a role in visualisation of the various steps in the process.

The ideal radiopharmaceutical to image inflammation should meet the following criteria: (1) rapid delineation of foci and extent of lession; (2) no significant physiological accumulation in the blood or organs such as liver, spleen, gastrointestinal tract, bone, bone marrow, kidneys; (3) rapid washout from background and retention in target; (4) discrimination between infection and non-microbial inflammation; (5) low toxicity and absence of immune response; and (6) low cost and ease of preparation (Corstens and van der Meer, 1999).

The radiopharmaceuticals routinely used for scintigraphic detection include ^{67}Ga-citrate (Staab and McCartney, 1978; Seabold et al., 1997a), ^{99m}Tc or ^{111}In labelled leukocytes (Seabold et al., 1997b), ^{99m}Tc-nanocolloid (Schrijver et al., 1987), and ^{99m}Tc or ^{111}In labelled human polyclonal immunoglobulin (HIG) (Buscombe et al., 1991; McAffe et al., 1991). Other preparations, mainly small receptor-specific proteins and peptides, are currently being tested. However, none of the preparations is capable of distinguishing, in a clinically helpful/useful manner, between infections and inflammatory lesions (Van der Laken et al., 1998).

Our research into new methods of labelling biologically active substances with technetium (^{99m}Tc) to be used in nuclear medicine is focused on low molecular peptides and proteins, which can be successfully labelled with ^{99m}Tc and will be useful for the detection and monitoring of inflammatory diseases.

The issue of labelling of proteins and peptides of labile structures is very extensive and complex, and has not been fully explored. Currently, small proteins and peptides are often employed whose structures in preparation and labelling are sensitive to reaction conditions. For this reason, we focused on the preparation of protein and peptide kits for labelling by technetium (^{99m}Tc) using redox polymers, which in our view, is the gentlest method (Komarek et al., 2000a). Use of redox polymers at pH values over 6.0 allows practically selective labelling of these compounds with technetium (^{99m}Tc) at end SH groups. Redox polymers do not require the use of other adjuvants to modify reaction conditions as is necessary with currently used methods (Kleisner et al., 2000).

2. Materials and methods

2.1. Study substances

Human leukocyte dialysate (HDL), IMMODIN-SEVAC, a.s., Prague; thymosin (TF5), fraction 5 from veal thymus, freeze-dried, SPOFA, a.s., Prague; aprotinin (Apt), SPOFA, a.s., Prague; human immunoglobulin (HIG), IgG-5% solution containing minimally 95% monomer and dimmer of IgG, NIGA-SEVAC, a.s., Prague.

2.2. Chemicals

Sodium pertechnetate (^{99m}Tc) Ultratechnekow FM Generator, Mallinckrodt Medical, BV; stannous chloride dihydrate (Merck); redox polymer (RP-IDA), FNKV Prague.

2.3. Preparation of the kits

Five milliliters of proteins or peptides (1–2% solutions) were mixed with 500 mg of the RP-IDA redox polymer. Kleisner et al. (2000) described the synthesis of the redox polymer with IDA functional groups from a dextran matrix. After stirring of 10% suspension for 16 h at room temperature, a mixture of the ligand with redox polymer was filtered (pore size 0.22 μm) and the solution dispensed into sterile vials (à 0.4 ml). All steps were performed in nitrogen atmosphere. Kits were stored at −35 °C.

2.4. Labelling with ^{99m}Tc

After reconstruction of the kit, 2 ml of sodium pertechnetate (^{99m}Tc) solution (50–100 MBq) was added. The solution was left to incubate for 20 min at room temperature.

2.5. Radiochemical purity assessment

The labelling efficiency was evaluated using paper chromatography on Whatmann No. 3. Acetone 90%

was used as a solvent. Additionally, ^{99m}Tc-labelled proteins were analysed by column chromatography on Sephadex G-50 (30×1) with saline as a solvent.

2.6. Biodistribution

The biodistribution of the labelled substances was evaluated in Wistar rats (body mass: 160–200 g). For quantitative determination of organ distribution, groups of five animals were sacrificed 4 h after the administration. Furthermore, the deposition into inflammatory tissue was determined. The administration of approximately 10^5 to 10^6 colony forming units of *Staphylococcus aureus* suspended in 0.2 ml of saline or 0.1 ml of 10% sterile kaolin suspension in saline into the left inguinal region induced the inflammation.

^{99m}Tc-labelled substances (0.1 ml; 370–500 kBq) were injected into tail vein 24–72 h thereafter. The radioactivity measured was expressed in percent of radioactivity administered in whole organs or as gram tissue. The distribution of radioactivity (20–40 MBq ^{99m}Tc-labelled substances) in the rats was analysed by scintillation gamma camera.

3. Results and discussion

For the kits prepared by means of redox polymers labelled with ^{99}Tc (Kleisner et al., 2000; Komarek et al., 1998, 2000a, 2000b), the radiochemical purity has been 97% at least. This corresponds with requirements set for routinely prepared radiopharmaceuticals. When labelling proteins or peptides with technetium (^{99m}Tc), the reductant employed is Sn^{2+} in the form of stannous chloride in acid medium (below pH 2.6). The drawbacks of this method include the possible inactivation of biologically active proteins and peptides as well as formation of colloids when adjusting pH to optimal values. The method using various complexes of Sn^{2+} to transfer this ion to protein or peptide also leads to protein/peptide 'contamination' with Sn^{2+} complexes with possible formation of colloids, which are practically unable to remove from the reaction mixture. It is for these reasons that we employ, when using biologically active substances for labelling with ^{99m}Tc, insoluble redox polymers on the basis of cross-linked dextran.

Use of redox polymers is intended especially for the labelling of labile structures and substances sensitive to reaction conditions. In these, the possibility of incorporating of chelating groups anchoring to proteins that can inactive them is virtually ruled out. As a result, labelling of proteins and peptides with technetium (^{99m}Tc) using redox polymers for use in nuclear medicine is most promising. This will provide the op-

Table 1
Labelling efficiency of the study substances

Time since labelling (h)	Labelling efficiency (%)			
	^{99m}Tc-HDL	^{99m}Tc-TF5	^{99m}Tc-Apt	^{99m}Tc-HIG
0	98.9	98.3	98.7	97.0
4	98.8	92.1	98.1	98.9
6	98.4	90.5	96.9	97.6
8	98.2	–	95.0	98.2
24	98.0	64.4	–	98.7
30	95.6	–	–	98.3

Table 2
Stability of the kits of study substances expressed as ^{99m}Tc labelling efficiency

Time since preparation (days)	Labelling efficiency			
	^{99m}Tc-HLD	^{99m}Tc-TF5	^{99m}Tc-Apt	^{99m}Tc-HIG
0	98.2	98.3	98.6	91.7
1	98.2	–	–	97.0
2	98.0	–	–	95.4
7	98.7	–	–	95.2
30	97.6	96.0	95.9	95.0
50	98.1	91.7	95.5	92.7
100	97.1	–	–	90.2
150	98.0	82.0	94.7	89.0
300	97.4	–	–	87.0

Table 3
Biodistribution of ^{99m}Tc-HIG [% i.d. ($x \pm$ S.D.) 4 h post injection]

Tissue	Organ	1g
Muscle-inflammation	–	0.29 ± 0.02 (K)
		0.32 ± 0.03 (S)
Muscle-control	–	0.03 ± 0.00
Blood	8.55 ± 0.77	0.48 ± 0.07
Lungs	0.74 ± 0.11	0.39 ± 0.09
Stomach	0.30 ± 0.07	0.14 ± 0.02
Liver	11.93 ± 0.92	1.49 ± 0.10
Spleen	0.26 ± 0.02	0.41 ± 0.07

(K), kaolin; (S), *Staphylococcus aureus*.

Table 4
Biodistribution of ^{99m}Tc-HLD [% i.d. ($x \pm$ S.D.) 4 h post injection]

Tissue	Organ	1 g
Muscle-inflammation	–	0.07 ± 0.01 (K)
		0.14 ± 0.03 (S)
Muscle-control	–	0.03 ± 0.00
Blood	2.19 ± 0.18	0.15 ± 0.01
Lungs	0.11 ± 0.08	0.15 ± 0.01
Stomach	0.77 ± 0.06	0.37 ± 0.02
Liver	1.00 ± 0.06	0.13 ± 0.01
Spleen	0.03 ± 0.00	0.05 ± 0.01
Kidney	10.93 ± 0.79	7.05 ± 0.78

(K), kaolin; (S), *Staphylococcus aureus*.

portunity to prepare radiopharmaceuticals in a kit form can be readily labelled with technetium (^{99m}Tc) attaining high radiochemical purity and stability.

When the labelling efficiency was evaluated, the highest stability for ^{99m}Tc-HIG and ^{99m}Tc-HDL proved to be within 30 h after preparation. For radiopharmaceuticals, routinely used in nuclear medicine, minimum stability time required is usually from 6 to 8 h. Stability of labelled substances with respect to the labelling efficiency is summed in Table 1.

Table 5
Biodistribution of ^{99m}Tc-Apt [% i.d. ($x \pm$ S.D.) 4 h post injection]

Tissue	Organ	1 g
Muscle-inflammation	–	0.04 ± 0.01 (K)
		0.08 ± 0.02 (S)
Muscle-control	–	0.03 ± 0.01
Blood	9.01 ± 1.70	0.68 ± 0.05
Lungs	0.67 ± 0.08	0.57 ± 0.05
Stomach	–	–
Liver	10.26 ± 0.77	1.17 ± 0.13
Spleen	0.59 ± 0.10	0.89 ± 0.08
Kidney	12.66 ± 1.12	8.32 ± 0.58

(K), kaolin; (S), *Staphylococcus aureus*.

Table 6
Biodistribution of ^{99m}Tc-TF5 [% i.d. ($x \pm$ S.D.) 4 h post injection]

Tissue	Organ	1 g
Muscle-inflammation	–	0.07 ± 0.02 (K)
		0.14 ± 0.03 (S)
Muscle-control	–	0.03 ± 0.01
Blood	3.75 ± 0.51	0.31 ± 0.05
Lungs	0.27 ± 0.01	0.26 ± 0.01
Stomach	–	–
Liver	2.92 ± 0.11	0.24 ± 0.03
Spleen	0.13 ± 0.04	0.32 ± 0.11
Kidney	0.21 ± 0.40	6.10 ± 0.35

(K), kaolin; (S), *Staphylococcus aureus*.

For all substances in focus, the method of preparation is aimed at the possible preparation of stock kits, which could be labelled with technetium ^{99m}Tc

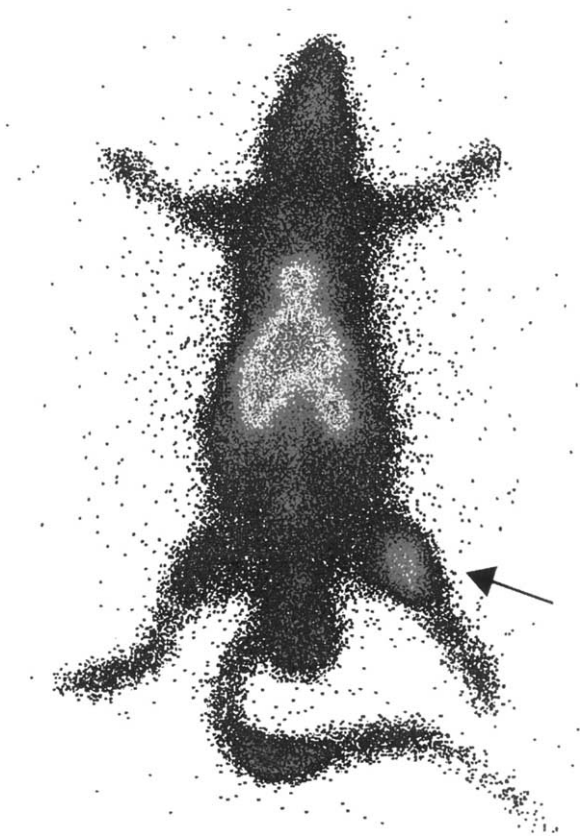

Fig. 1. Distribution of activity in the rat 4 h after injection of ^{99m}Tc-HIG.

Table 7
Radioactivity in experimentally induced inflammation

Labelled substance	Type of inflammation	Ratio of activity (inflammation/control)	
		Radioactivity counting (% i.d. in 1 g of isolated tissue)	Gamma camera imaging (pixels)
^{99m}Tc-HIG	Kaolin	9.7	8.8
	Staphylococcus	10.6	9.3
^{99m}Tc-HDL	Kaolin	2.3	1.8
	Staphylococcus	4.7	3.8
^{99m}Tc-Apt	Kaolin	1.2	2.0
	Staphylococcus	2.6	2.5
^{99m}Tc-TF5	Kaolin	2.2	2.4
	Staphylococcus	4.6	3.8

yielded from a ^{99}Mo–^{99m}Tc generator. This enables to employ such kits when required for diagnostics. Stability of the kits of study substances expressed as ^{99m}Tc labelling efficiency is shown in Table 2. In accordance with data in Table 1, the highest stability within 300 days since preparation of the kits has been proven for kits specified for ^{99m}Tc labelling human leucocyte dialysate (^{99m}Tc-HLD) and human immunoglobulin (^{99m}Tc-HIG). However, all the study substances may be prepared as stock kits with a minimum shelf life of 30 days in case of ^{99m}Tc-TF5, longer in case of remaining kits (^{99m}Tc-Apt, ^{99m}Tc-HLD) (see Table 2).

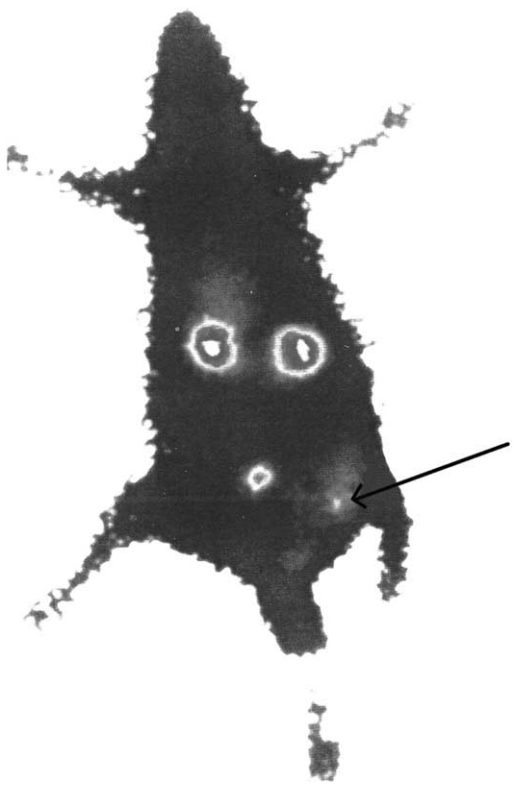

Fig. 2. Distribution of activity in the rat 4 h after injection of ^{99m}Tc-HDL.

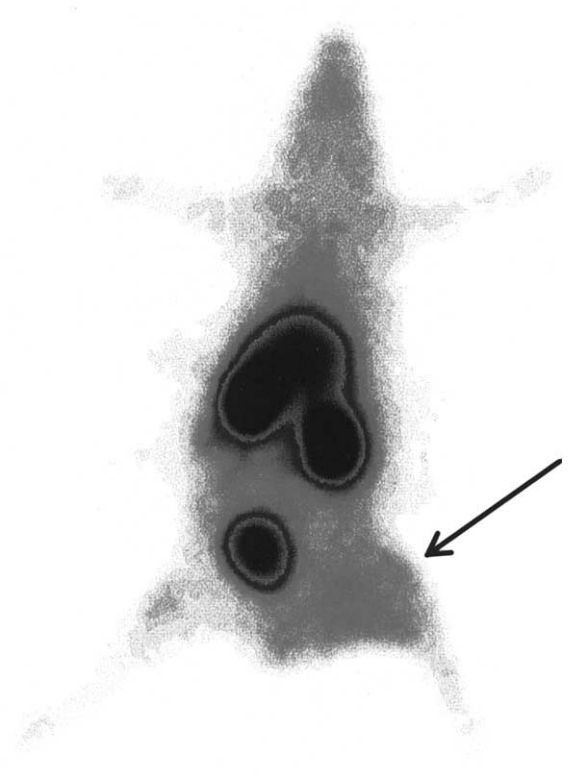

Fig. 3. Distribution of activity in the rat 4 h after injection of ^{99m}Tc-Apt.

Suitability of respective substances for inflammation process imaging has been confirmed in biodistribution studies on rats. The distribution of radioactivity in rat organs and inflammation tissues as measured in vitro is shown in Table 3 (^{99m}Tc-HIG), Table 4 (^{99m}Tc-HLD), Table 5 (^{99m}Tc-Apt) and Table 6 (^{99m}Tc-TF5). When comparing data in aforementioned Tables, ^{99m}Tc-HIG exhibits the highest activity in inflammatory foci although the remaining study substances could be considered as potential radiopharmaceuticals as well.

Diagnostics practice requires, however, a determination of inflammatory disorder with further differentiation between infection foci and other types of inflammation. Table 7 shows the values of radioactivity distribution in inflammation foci compared to the control tissue in so-called sterile inflammation (an inflammation induced by kaolin suspension) and infection induced by *S. aureus*. In all cases, infection foci accumulated higher amounts of labelled study substances. Labelled peptides incorporated double amounts of activity. The highest amounts of activity in both types of inflammation have been determined after administering labelled protein (^{99m}Tc-HIG). Activity per unit of selected area in experimentally induced inflammation was measured by means of scintigraphic imaging. Such imaging method enabled direct comparison between the control and the inflammation tissue (Table 7). This is clearly visible in whole body imaging of rats, where the areas of inflammation induced by *S. aureus* are marked with an arrow (see Figs. 1–4).

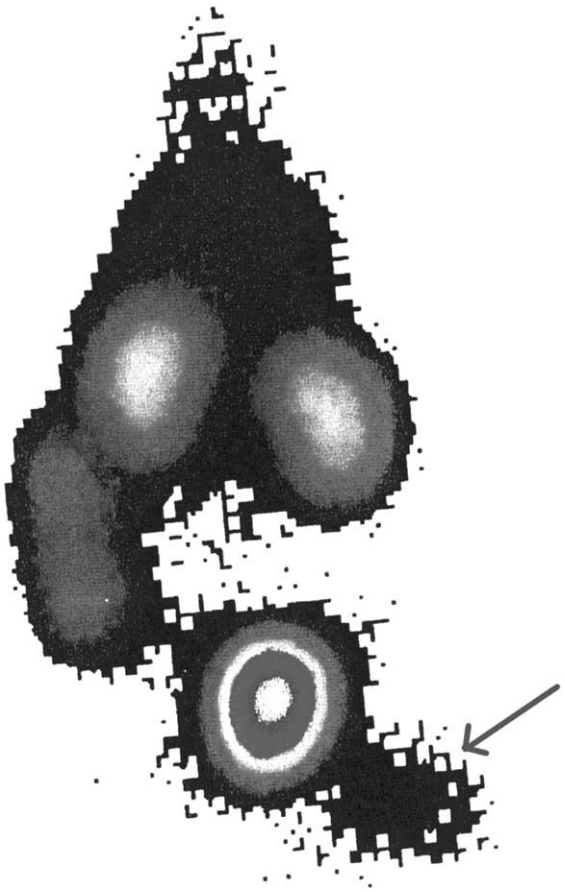

Fig. 4. Distribution of activity in the rat 4 h after injection of ^{99m}Tc-TF5.

4. Conclusion

The method for preparing protein- and peptide-containing kits using RP-IDA allows effective and stable labelling of the study substances with technetium (^{99m}Tc). The evaluated peptides and proteins show a high labelling efficiency (minimum 95%), which remains to be virtually the same (95–98%) after 8 h at least. In experimentally induced inflammation, the amount of ^{99m}Tc-peptides and proteins activity accumulated is 2.5–6.5 and 5.3–10.6 times, respectively, higher, than in control tissue. A comparison of two types of model inflammations (inflammation induced by kaolin and *Staphylococcus*-induced inflammation) revealed that the values measured with ^{99m}Tc-peptides are more than a double than those induced by kaolin suspension. The studied low molecular peptides labelled with ^{99m}Tc allow rapid localisation of infection foci in an animal model. ^{99m}Tc labelled HIG seems to be useful for the detection of both infections and inflammatory lesions.

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References

Buscombe, J.R., Miller, R.F., Lui, D., 1991. Combined Gallium-67-citrate and ^{99m}Tc-human immunoglobulin imaging in human immunodeficiency virus-positive patients with fever of unknown origin. Nucl. Med. Commun. 12, 583–592.

Corstens, F.H., van der Meer, J.W., 1999. Nuclear medicine's role in infection and inflammation. Lancet 354, 765–770.

Kleisner, I., Komarek, P., Komarkova, I., Konopkova, M., 2000. The use of redox polymers in labelling procedures of proteins and peptides with ^{99m}Tc: I. Properties of redox polymers and technique of labeling. Nucl. Med. Rev. 3, 65–68.

Komarek, P., Kleisner, I., Konopkova, M., Komarkova, I., 1998. Photoactivated proteins labelled with technetium-99mTc using redox polymers: a new approach to radiopharmaceutical preparation. Eur. J. Pharm. Sci. 6 (Suppl. 1), 23 (abstract).

Komarek, P., Kleisner, I., Komarkova, I., Konopkova, M., 2000a. The use of redox polymers in labelling procedures of proteins and peptides with ^{99m}Tc: II. Technique of preparation of kits for protein labelling by ^{99m}Tc and its effect on the stability and radiochemical purity. Nucl. Med. Rev. 3, 69–72.

Komarek, P., Kleisner, I., Komarkova, I., 2000b. A simple method of labelling some proteins and peptides with ^{99m}Tc using a monocomponent kit. Eur. J. Nucl. Med. 27, 216 (abstract).

McAffe, J.G., Gagne, G., Subramanian, G., 1991. The localisation of indium-111-leukocytes, galium-67, polyclonal IgG and other radioactive agents in acute focal inflammatory lesions. J. Nucl. Med. 32, 2126–2131.

Schrijver, M.D., Streule, K., Senekowitsch, R., 1987. Scintigraphy of inflammation with nanometer-sized colloidal tracers. Nucl. Med. Commun. 8, 895–908.

Seabold, J.E., Palestro, C.J., Brown, M.L., 1997a. Procedure guideline for gallium scintigraphy in inflammation. J. Nucl. Med. 38, 994–997.

Seabold, J.E., Forstrom, L.A., Schauwecker, D.S., 1997b. Procedure guideline for indium-111-leukocyte scintigraphy for suspected infection/inflammation. J. Nucl. Med. 38, 997–1001.

Staab, E.V., McCartney, H., 1978. Role of ^{67}Gallium in inflammatory disease. Semin. Nucl. Med. 8, 219–234.

Van der Laken, C.J., Boerman, O.C., Oyen, J.G., 1998. Scintigraphic detection of infection and inflammation: new developments with special emphasis on receptor interaction. Eur. J. Nucl. Med. 25, 535–546.