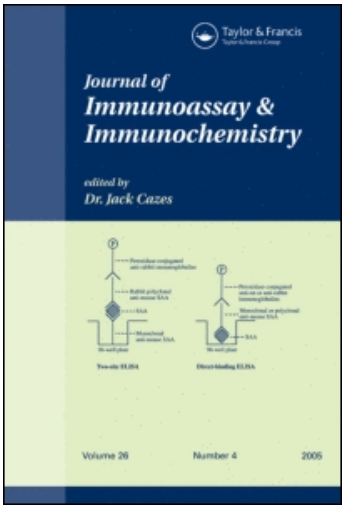

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Jonathan Patinkin[a]; Dan Inbar[a]; Chana Ben-gigi[a]; Sara Derfler[a]; Yakir Klausner[a]; Bertold Fridlender[a]
[a] Department of Research and Development, Ames-Yissum Ltd., Jerusalem, Israel

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HOMOGENEOUS SUBSTRATE-LABELED FLUORESCENT IMMUNOASSAY FOR HUMAN SERUM ALBUMIN

Jonathan Patinkin, Dan Inbar, Chana Ben-Gigi, Sara Derfler,
Yakir Klausner and Bertold Fridlender
Ames-Yissum Ltd.
Department of Research and Development
Jerusalem, Israel

ABSTRACT

A homogeneous substrate-labeled fluorescent immunoassay for human serum albumin (HSA) has been developed, similar to previously described immunoassays for Immunoglobulin G and Immunoglobulin M. HSA was covalently linked to 6-(7-β-galactosylcoumarin-3-carboxamide) hexylamine. The resulting conjugate had minimal fluorescence at 450 nm (with excitation at 400 nm). However, when the acetal linkage of the galactosyl moiety was hydrolyzed by β-galactosidase, a substantial increase in the fluorescence was obtained. This increase was specifically inhibited by antibody to HSA. A competitive binding immunoassay was established by letting the conjugate compete with HSA in the serum for the limited number of antibody-binding sites. The level of fluorescence resulting from the addition of enzyme was proportional to the amount of HSA in the serum. Precision, analytical recovery and serum dilution studies were carried out on the assay. The immunoassay was compared to an albumin assay using the dye-binding method.

INTRODUCTION

Human serum albumin (HSA) is the major protein in the plasma, comprising 50-60% of the total plasma protein (1,2). It is thought to have three major physiological functions. These are colloidosmotic regulation, transport and nutrition. A normal albumin level portends a state of well-being in contrast to the many disease states which are accompanied by a fall in albumin levels. Several methods have been developed for assaying albumin in the serum, but the most

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common ones remain dye-binding methods (3) and radial immunodiffusion (4).

Recently, several homogeneous immunoassays for proteins have been described in the literature (5). Among these, two are based on the principle of substrate labeled fluorescent immunoassay (SLFIA), one for immunoglobulin G (IgG) (6), and one for immunoglobulin M (IgM) (7). In these assays, a fluorogenic enzyme substrate, N-(6-Aminohexyl)-7-(β-O-galactopyranoside) Coumarin-3-Carboxamide (GUAH) is covalently linked to the protein. This protein-substrate conjugate can be hydrolyzed by β-galactosidase at the galactose-coumarin ester linkage to yield a product which is highly fluorescent at excitation and emission wavelengths of 400 and 450 nm, respectively. When antibody specific to the protein is bound to the conjugate, this hydrolysis is substantially reduced. As in other immunoassays of this type, the introduction of unlabeled protein into the system (i.e. serum) brings about a competition for the limited number of antibody-binding sites between the labeled and unlabeled protein. An increase in the amount of unlabeled protein causes a proportional reduction in the amount of conjugate bound to the antibody with a concomitant increase in enzyme-induced fluorescence. Thus, a fluorescent signal is obtained without the need to physically separate the unbound and antibody-bound labeled antigen.

In this communication we describe a homogeneous immunoassay for human serum albumin using the SLFIA technique.

MATERIALS AND METHODS

Synthesis of Fluorogenic Substrate

GUAH was synthesized as previously described (7).

Coupling of Fluorogenic Substrate to HSA

In a typical coupling experiment, 3.5 mg of dimethyladipimidate (DMA) (14.4 umoles) (Pierce; Rockford, Ill.) were dissolved in 400 µl of 0.5 M K_2CO_3 pH 9.6 immediately prior to the start of the reaction and 6 mg of GUAH (12 µmoles) were dissolved in 400 µl of water. The GUAH solution was added to the DMA solution and the mixture stirred for 5 minutes at room temperature. Then, 8 mg of HSA (0.12 µmoles) (Calbiochem-Behring; La Jolla, Calif.) in 600 µl of 0.1 M sodium pyrophosphate pH 9.0 were added, and the mixture stirred for a further 60 minutes. The solution was then loaded on a 1.1 x 22 cm column of Sephadex G-50 (fine) equilibrated with 0.1 M sodium phosphate buffer pH 7.0. 25 fractions of 1.0ml each were collected, and their absorbance at 280 and 340 nm measured. In addtion, 4 µl from each fraction were spotted on a strip of Whatman filter paper which was stained with 0.2% Commaissie brilliant blue in 10% acetic acid and subsequently destained with 10% acetic acid. The first peak of absorbance corresponding to the stained fractions on the filter paper were pooled and dialyzed against the following buffers at 4^0C: 1) 3 liters of 0.1 M sodium phosphate pH 6.0 + 1.0 M NaCl (24 hours); 2) 3 liters of 0.05 M citric acid / 0.1 M Na_2HPO_4 pH 5.5 + 1 M NaCl (20 hours); 3) same buffer as in (2) at pH 4.6 without NaCl (20 hours); 4) same buffer as in (3) + 0.1% NaN_3 (7 hours).

The GUAH and conjugate were protected from direct light at all times during the coupling process. The labeled protein was stored in a brown bottle at 4^0C and was stable for at least 4 months.

Substrate Labelled Fluorescent Immunoassay for HSA

All dilutions were carried out with 20 mM N, N-bis (2-hydroxyethyl) glycine buffer (BICINE), pH 8.2, containing 25 mM $MgCl_2$ and

0.1% NaN_3 using a Cavro pipettor-dilutor. Unknown sera were diluted 1000-fold. Standards were prepared from 100% pure (electrophoresis) HSA (Calbiochem-Behring Corp.; La Jolla, Calif.) diluted in buffer. The HSA concentration of the stock solution was verified spectrophotometrically ($\varepsilon_{278\ nM}^{1\ mg/ml} = 0.53$) Standards equivalent to 0-65 g/l were prepared which more than cover the normal range of albumin levels found in serum (35-50 g/l). The actual concentrations of the standards were 0,15,30,40,50 and 65 mg/L. The GU-HSA conjugate, goat anti-human albumin (Kallestad Laboratories, Inc.; Chaska, Mn.), and β-galactosidase (Sigma Co.; St. Louis, Mo.) were pre-diluted so that 125 µl of the diluted solutions contained 0.037 µmoles, 4 µl and 1.0 unit (defined below) respectively. The dilutor was adjusted to dispense 250 µl of buffer together with each delivery of reagent. The reagents were added to a series of plastic cuvettes (Evergreen Scientific; Los Angeles, Calif.) in the following order: 1) standard or serum, 2) fluorescent conjugate, and 3) antiserum. No incubation period is necessary, since equilibrium is reached in less than one minute. The end-point reaction was initiated by the addition of β-galactosidase to successive cuvettes at 0.5 minute intervals. Each cuvette was mixed following the addition of enzyme and the cuvettes were incubated for 20 min. at room temperature. At the completion of the incubation time the fluorescence of each cuvette was measured and a standard curve was prepared by plotting fluorescence vs. HSA concentration. The albumin concentrations of the unkown sera were calculated from the standard curve by multiplying the resulting concentration by the dilution factor (1000).

Bromocresol Green Assay

20 µl of unknown sera or HSA standard were added to 3.0 ml of bromocresol green (BCG) solution (Miles Laboratories; Milano, Italy).

After a 1 min. incubation at room temperature the absorbance of the solution was measured at 628 nm and the concentration of albumin was calculated from the absorbance of the standard.

β-Galactosidase Activity

1 unit of β-galactosidase activity is defined as the amount of enzyme which hydrolyzes 1 μmole of orthonitrophynyl-β-O-galacto-pyranoside per min. in 50 mM Bicine buffer, pH 8.2 at 25°C (8).

Aborbance and Fluorescence Measurements

Fluorescence intensities and spectra were recorded at room temperature with an Aminco-Bowman spectrophotofluorometer (American Instrument Co.; Silver Spring, MD.) or an Ames fluoro-colorimeter. When cuvettes from the immunoassay were measured in the spectrophotofluorometer, the excitation and emission monochromators were set at 400 nm and 450 nm respectively. Optical absorption spectra were recorded with a Beckman DU-8 spectrophotometer.

RESULTS

Coupling of Fluorescent Substrate to HSA

The reaction by which GUAH is coupled to HSA is influenced by the various parameters of the reaction, such as pH, time, and the molar ratio of the reactants. These parameters can affect both the number of labels per protein and the yield of protein. When the molar ratio of DMA to GUAH was varied from 0.6 to 6 it was found that a ratio of 4 gave the highest number of labels per protein without affecting the yield (Figure 1). For routine syntheses it was decided to work with a ratio of 1.2 since this minimized the possibility of cross-linking the albumin while retaining an acceptable level of label density.

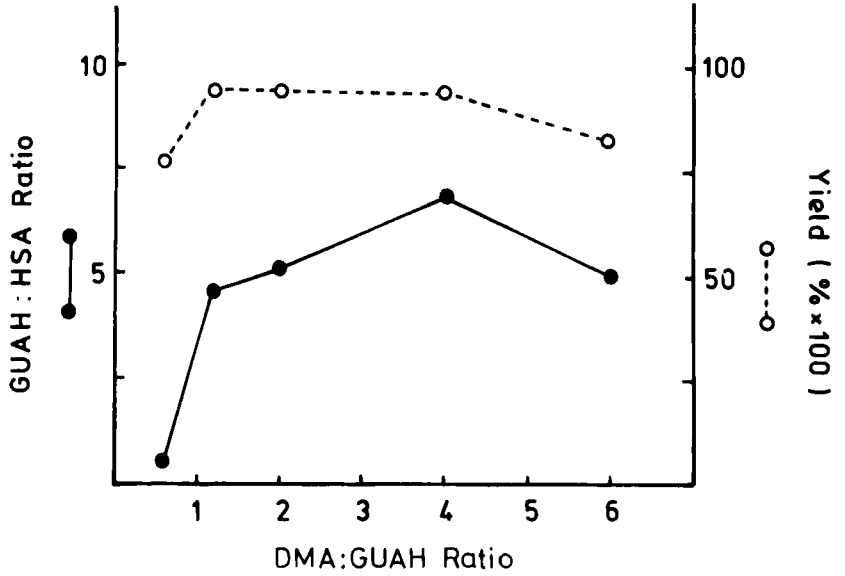

FIGURE 1. Effect of DMA:GUAH Molar Ratio on the Coupling of GUAH to HSA. The coupling reaction was carried out as described in Materials and Methods with 4mg of HSA and a corresponding reduction in the amounts of the other reactants. The concentration of the DMA solution was varied to obtain the different molar ratios. The yield was calculated by dividing the amount of protein in the first Sephadex peak by the amount of HSA added to the reaction, multiplied by 100.

Optical Properties of Fluorescent Labelled Albumin

GU-HSA, like other galactosyl-umbelliferone protein conjugates, has an optical absorption spectrum with two maxima, one at 340 nm due to the GUAH label (8) and one at 278 nm due to the protein with contributing absorption by the label. When GU-HSA is treated with β-galactosidase, the acetal linkage of the galactosyl moiety is hydrolyzed and a new absorption maxima appears at 405 nm, while the 340 nm maxima decreases. From the difference in absorption at 340 nm before and after hydrolysis, it can be calculated that approximately 25% of the label can be hydrolyzed by β-galactosidase. When the hydrolysis reaction is carried out under the

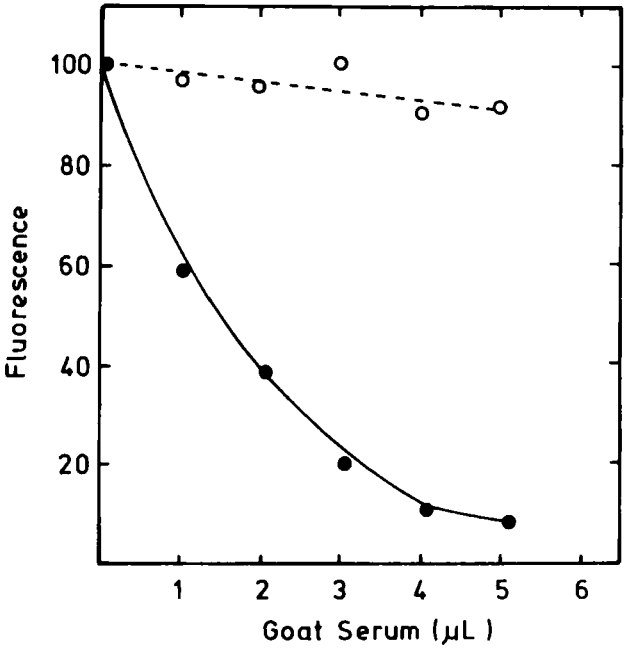

FIGURE 2. Effect of Goat Antiserum to HSA on Hydrolysis of GU-HSA by β-Galactosidase. To a series of tubes containing 100 µl of GU-HSA (3.1 µg) were added the amounts of goat antiserum (●) or normal goat serum (o) indicated in the figure. The tubes were incubated for 10 minutes and then 3.0 ml of 20 mM Bicine buffer containing 25 mM $MgCl_2$ and 0.1% NaN_3, pH 8.2 were added and the fluorescence of the tubes was measured. Then 100 µl of buffer containing 2 units of β-galactosidase was added, and the reaction was allowed to proceed at room temperature for 20 minutes. The fluorescence was measured and the background fluorescence previously measured was subtracted. The net fluorescence is presented as a percentage of the fluorescence of the normal goat serum extrapolated to 0 µl serum.

conditions of the immunoassay, 50 to 60% of the exposed labels are hydrolyzed within 20 minutes.

Before enzyme hydrolysis, GU-HSA has fluorescence excitation and emission maxima at 350 nm and 395 respectively. After enzyme hydrolysis, the product has excitation and emission maxima at 405 nm and 450 nm respectively. At an excitation wavelength of 400 nm and emission wavelength of 450 nm, the unhydrolyzed

conjugate has less than 5% of the fluorescent intensity of the hydrolyzed product. This provides a means of separating between hydrolyzed and unhydrolyzed conjugate in the same test tube.

Titration of Antibody to HSA with Fluorescent Labelled Albumin

The antigenicity of HSA is not affected by its coupling to GUAH, as evidenced by immunoelectrophoresis with anti-HSA antiserum. Furthermore, the binding of the conjugate by antibody interferes with the hydrolysis of the conjugate by β-galactosidase. This can be seen in an experiment in which a constant amount of enzyme was added to a fixed amount of GUAH-HSA and increasing concentrations of antiserum. The fluorescence produced by the enzymatic reaction decreased as the antiserum level increased, while normal goat serum had practically no effect (Figure 2).

Competitive Binding Immunoassay

From the previously described characteristics of the antibody and conjugate, it was evident that a homogenous competitive binding immunoassay could be established by letting the conjugate compete with a series of HSA standards or sera for sites on the antibody. The immunoassay was performed as described in Materials and Methods, and a typical standard curve is depicted in Figure 3. Satisfactory standard curves were obtained with enzyme incubations ranging from 6 to 30 minutes, provided each cuvette was incubated for the same amount of time. The standards were prepared on the assumption of a 1000-fold dilution of serum. A standard curve based on a 100-fold dilution of serum could be constructed, but was less satisfactory due to the large amounts of albumin.

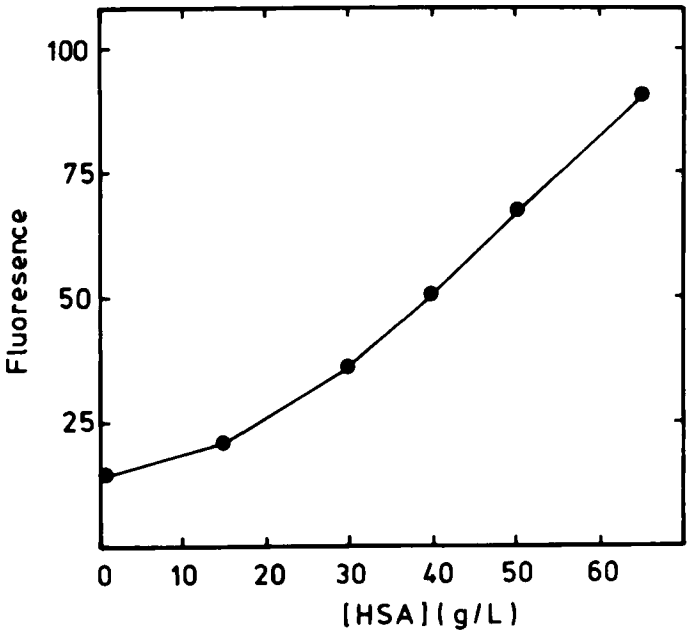

FIGURE 3. Standard Curve for HSA. The assay was carried out as described in Materials and Methods. The standards were prepared to be in the range of the amount of albumin in human serum diluted 1000-fold. The HSA concentrations on the abscissa correspond to the concentration of HSA in the undiluted serum.

Detection Limit

Solutions containing low concentrations of HSA were assayed together with the standard curve in order to ascertain the minimum concentration which is significantly different from zero as determined by the Mann-Whitney test. The minimum detectable concentration of HSA was 10 g/l which is sufficient for clinical purposes since levels of 15-18 g/l are considered extremely low (9).

Precision Studies

The intra-assay precision was estimated by assaying 15 replicates each of sera containing low, normal and high concentrations of HSA in

TABLE 1

Precision of SLFIA for HSA

	Serum sample g/l		
	Low	Normal	High
Intra-day			
Range	13.3 - 22.2	33.2 - 35.5	40.7 - 51.3
Mean	18.1	34.1	47.2
S.D.	2.43	0.71	2.95
CV%	13.4	2.1	6.2
N	15	15	15
Inter-day			
Range	14.2 - 21.8	34.1 - 42.2	51.0 - 67.0
Mean	18.7	37.2	58.9
S.D.	2.1	2.6	4.7
CV%	11	7	8
N	20	20	20

the same experiment. The inter-assay precision was determined by assaying 20 replicates of the same sera over a period of two weeks. The CV's ranged from 2 to 13% for the intra-assay and 7 to 11% for the inter-assay studies (Table I).

Analytical Recovery

Sera with known concentrations of HSA were mixed in various combinations and analyzed by the SLFIA and BCG methods. The results presented in Table II show that the values determined by the two methods agree reasonably well.

Serum Dilution Studies

The basic assay format includes a 1000-fold predilution of the sera in bicine buffer which should preclude any serum effects on the assay. To ensure that this was so, serum dilution studies were carried

TABLE 2

Analytical Recovery of HSA in SLFIA and BCG

	Conc. g/L of HSA		Expected value g/L		Observed value		Recovery OBS/EXP	
Serum	SLFIA	BCG	SLFIA	BCG	SLFIA	BCG	SLFIA	BCG
1	61.5	50.2						
2	35.6	33.0						
3	57.9	44.0						
4	20.9	19.0						
5	18.1	18.8						
1 + 3			59.7	47.1	55.7	45.5	0.93	0.97
4 + 5			19.5	18.9	17.8	18.1	0.91	0.96
1 + 4			41.2	34.6	37.7	34.5	0.92	1.00
2 + 3			46.7	38.5	48.3	37.3	1.03	0.97

out in which sera were pre-diluted to higher or lower dilutions and the results multiplied by the appropriate dilution factor. The only restriction on dilution was that the results should fall within the standard curve. The results show that the serum effect is minimal (Table III).

Correlation of Substrate Labelled and Dye Binding Assay

The albumin concentrations of 58 individual sera were determined by the SLFIA and BCG methods (Figure 4) and ranged from 17.9 to 57 g/l. The regression line for the results is described by the equation SLFIA = 1.15 (BCG) + 1.26 with a correlation coefficient of 0.91 and a standard error of 3.7 g/l.

DISCUSSION

We have developed a SLFIA for HSA similar to those previously described for other proteins. Various methods are used to quantitative

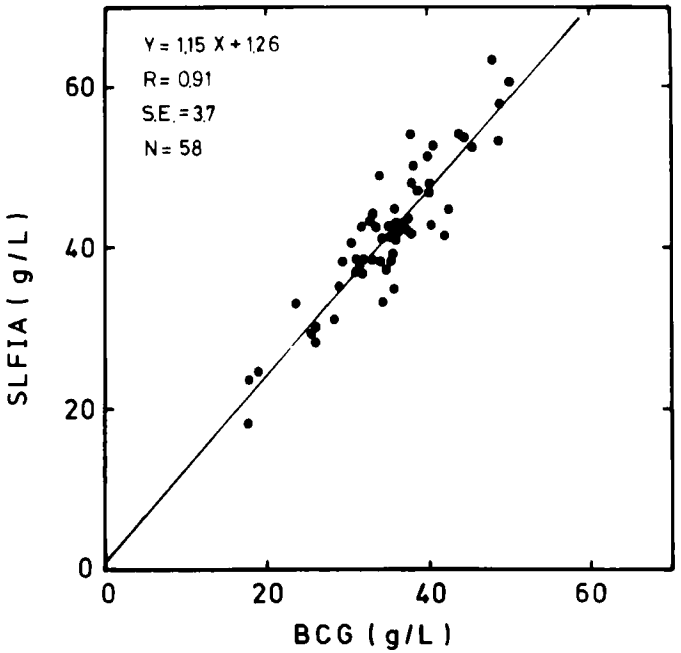

FIGURE 4. Correlation of HSA concentration measured in human sera by SLFIA and BCG assays.

albumin in the serum, including electrophoresis, precipitation and dye binding methods. Of the aforementioned, the dye-binding methods are the most popular and adaptable to automation. Among the dyes used are methyl orange, 8-anilino-1-naphthalene sulfonic acid (ANS), 2-(4-hydroazobenzene) benzoic acid (HABA) and BCG. BCG is the most commonly used dye since it is least given to bind to other serum proteins and the method is simple, rapid, and inexpensive. (10). However, albumin levels are routinely over-estimated by the BCG method, especially in the clinically crucial lower concentration range (11), and although certain modifications have been introduced, the accuracy of the method leaves room for improvement.

TABLE 3

Effect of Serum Dilution on SLFIA Results

	HSA conc. g/L* with serum dilution of										
Serum	250	500	750	1000	1500	2000	3000	5000	Mean	S.D.	CV (%)
1	16.8	19.1	19.3	16.8	17.1				17.8	1.27	7.1
2		23.9		23.2	25.8	20.3			23.3	2.28	9.8
3		31.2		34.1	33.4	29.1			31.9	2.27	7.1
4			40.5	44.9	43	42.7	42.7		42.8	1.56	3.7
5			46.8	49.2	53.1	46.9	49		49	2.56	5.2
6				56.7		57.9		53.7	53.6	5.26	9.8

*Observed values were multiplied by appropriate dilution factor.

Among immunological methods for the measurement of albumin, radial immunodiffusion (RID) is the major method used in clinical laboratories. Although more accurate than the BCG method, it is difficult to precisely measure the diameters. The assay takes longer and is not suitable to automation. The latter is also true of manual nephlometric techniques, in addition to the added complication of the need to filter all the solutions. Automated immunoprecipitation (AIP) is found in relatively few clinical laboratories due to the high cost of the equipment and reagents and the need for skilled personnel with experience in continuous flow systems.

A non-separation fluoroimmunoassay for HSA has been described using an "indirect quenching" method.[12] although this method has good precision and correlates fairly well with an automated immunoprecipitation method, it uses an increased amount of serum sample, thereby requiring a separate blank mixture for each sample to correct for the intrinsic fluorescence of the serum. In addition, two antisera are used requiring two separate titrations.

The SLFIA for HSA described here combines the accuracy and reliability of the RID method with the simplicity and convenience of the BCG method. It has good precision, minimal serum effects, and correlates well with the BCG method. Its accuracy extends to the lower concentration range. This, together with the possibility of automation, should make this a desirable assay for the measurement of albumin in serum.

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Reprint Requests should be addressed to Jonathan Patinkin, Ames-Yissum Ltd., Jerusalem, Israel.

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