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Synthesis of sialyl Lewis^a (sLe^a, CA19-9) and construction of an immunogenic sLe^a vaccine

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Abstract

Sialyl Lewis^a (sLe^a), also termed CA19-9 antigen, is recognized by murine mAb19-9 and is expressed on the cancer cell surface as a glycolipid and as an O-linked glycoprotein. It is highly expressed in a variety of gastrointestinal epithelial malignancies including colon cancer and pancreatic cancer, and in breast cancer and small cell lung cancer, but has a limited expression on normal tissues.

sLe^a is known to be the ligand for endothelial cell selectins suggesting a role for sLe^a in cancer metastases and adhesion. For these reasons, sLe^a may be a good target for antibody mediated immunotherapy including monoclonal antibodies and tumor vaccines. However, sLe^a is structurally similar to sLe^x and other blood group related carbohydrates which are widely expressed on polymorphonucleocytes and other circulating cells, raising concern that immunization against sLe^a will induce antibodies reactive with these more widely expressed autoantigens. We have shown previously both in mice and in patients that conjugation of a variety of carbohydrate cancer antigen to keyhole limpet hemocyanin (KLH) and administration of this conjugate mixed with saponin adjuvants QS-21 or GPI-0100 are the most effective methods for induction of antibodies against these cancer antigens. We describe here for the first time the total synthesis of pentenyl glycoside of sLe^a hexasaccharide and its conjugation to KLH to construct a sLe^a-KLH conjugate. Groups of five mice were vaccinated subcutaneously four times over 6 weeks. Sera were tested against sLe^a-HSA by ELISA and against sLe^a positive human cell lines adenocarcinoma SW626 and small cell lung

cancer (SCLC) DMS79 by FACS. As expected, mice immunized with unconjugated sLe^a plus GPI-0100 or unconjugated sLe^a mixed with KLH plus GPI-0100 failed to produce antibodies against sLe^a. However, mice immunized with sLe^a-KLH conjugate without GPI-0100 produced low levels of antibodies and mice immunized with sLe^a-KLH plus GPI-0100 produced significantly higher titer IgG and IgM antibodies against sLe^a by ELISA. These antibodies were highly reactive by FACS and mediated potent complement mediated cytotoxicity against sLe^a positive SW626 and DMS79 cells. They showed no detectable cross reactivity against a series of other blood group-related antigens, including Le^y, Le^x, and sLe^x by dot blot immune staining. This vaccine is ready for testing as an active immunotherapy for treating sLe^a positive cancer in clinical settings.

Keywords

Sialyl Lewis^a; CA19-9; Carbohydrate conjugate vaccine; Cancer vaccine

Introduction

Sialyl Lewis^a (sLe^a) [NeuAcα2→3Galβ1→4(Fucα1→3) GlcNAcβ1→3Galβ-] [1] is recognized by murine monoclonal antibody 19-9 [2]. The sLe^a antigen (also termed CA19-9) is expressed as a glycolipid with the ceramide moiety imbedded in cell membrane lipid bilayers or as a glycoprotein, most commonly O-linked on mucins. The sLe^a expression on normal tissues is restricted to ductal epithelial in the lumen of a variety of epithelial tissues, sites largely inaccessible to antibodies and antibody mediated effector mechanisms. It is, however, widely expressed on a variety of gastrointestinal epithelial malignancies [3] including colon cancer [4] and pancreatic cancer [5], but also on breast cancer [6] and small cell lung cancer (SCLC), [3] indicating a higher frequency of nodal involvement and a worse prognosis [4,6,7] and when sLe^a is detected in the serum, it serves as a marker for these malignancies. sLe^a is also known as a ligand for leukocyte endothelial adhesion molecules such as endothelial E-selectin [8-10]. The fact that sLe^a is expressed as a carbohydrate epitope on glycolipids or glycoproteins make it an unlikely target for classical cell mediated immunity based therapeutic interventions. However, the high expression of sLe^a at the cell surface on several epithelial malignancies, its limited expression on normal tissues that are accessible to the immune system and its possible role in the metastatic process makes sLe^a a good target for antibody mediated immunotherapy including monoclonal antibodies and antibody inducing tumor vaccines.

The preparation of vaccines against defined autoantigens such as sLe^a is difficult because induction of active immunity against them involves overcoming some degree of immune tolerance. In our experience the most effective method for inducing antibodies against defined autoantigens such as sLe^a has been conjugation of the antigen to an immunogenic protein carrier molecule such as keyhole limpet hemocyanin (KLH) [11] and administration of the resulting conjugate combined with a saponin immune adjuvant such as the purified saponin fraction QS-21 [12] or the semi-synthetic saponin mixture GPI-0100 [13]. KLH is a large highly immunogenic protein purified from the blood of the keyhole limpet. GPI-0100 has been chemically modified to decrease toxicity and improve stability [14]. We have shown previously in mice and in patients that this combination approach is the most effective method for induction of antibodies against a variety of gangliosides, neutral glycolipids and other carbohydrate and peptide antigens (reviewed in [15]).

There are no previous reports describing vaccines composed of sLe^a, largely because of the lack of natural sources of easily extractable sLe^a and the difficulties encountered in chemically synthesizing such a complex molecule. We describe here for the first time the total chemical synthesis of sLe^a based on the one pot synthesis technology (OPopS) [16], and its conjugation to KLH to construct sLe^a-KLH. We report herein that the sLe^a-KLH conjugate plus GPI-0100

vaccine induces high titer IgM and IgG Abs which are highly specific for sLe^a and react strongly with sLe^a positive cancer cells.

Materials and methods

Antigens and reagents

Keyhole limpet hemocyanin (KLH) was purchased from Sigma Chemical Company (St Louis, MO). Human serum albumin (HSA), sodium cyanoborohydride, and human serum compliment were also obtained from Sigma Chemical Company. 4-(4-*N*-Maleimidomethyl) cyclohexane-1-carboxyl hydrazide (MMCCH) was purchased from Toronto Chemicals (Toronto, Canada). Monoclonal antibody 121SLE recognizing sLe^a was purchased from GeneTex, Inc. (San Antonio, TX). Goat anti-mouse IgG and IgM conjugated with alkaline phosphatase and goat anti-mouse IgG and IgM fluorescence-isothiocyanate (FITC) were obtained from Southern Biotechnology Associated (Birmingham, AL). Adjuvant GPI-0100, a semi-synthetic mix of saponin fractions was obtained from Dante Marciali, Galenica Pharmaceuticals, Inc. (Birmingham, AL), now known as Hawaii BioTechnology Inc. (Honolulu, Hawaii). SLe^a-PAA, sLe^x-PAA, Le^x-PAA and Le^y-PAA were purchased from Glycotect Corp., Gaithersburg, MD. Globo H-Ceramide, Le^y-Ceramide STn(c)-HSA, Tn(c)-HSA and Poly SA-HSA were prepared by our group as described in [15].

Preparation of sLe^a and sLe^a-KLH conjugate

The pentenyl glycoside of sLe^a hexasaccharide was synthesized by Optimer Pharmaceuticals, Inc. (San Diego, CA) using their glycosylation (OPopS) technology [16] starting from suitably protected mono, di and trisaccharide intermediates as described in Fig. 1. The product was characterized after each step using NMR spectroscopy in combination with mass spectroscopy. The ¹H-NMR spectrum of the final sLe^a pentenyl glycoside in deuterated water was fully consistent with the sLe^a structure. The pentenyl group was used for conjugation to KLH essentially as described in Fig. 2 [17]. The sLe^a-KLH conjugation procedure was initiated by ozonolytic cleavage of the pentenyl double bond and the resulting aldehyde was reacted with the hydrazine group of the MMCCH in the presence of sodium cyanoborohydride to give the sLe^a-MMCCH. KLH was treated with 2-iminothiolane to introduce thiol groups through the lysine ε-amino groups of the KLH to afford the thiolated KLH. Finally, the sLe^a-MMCCH and the thiolated KLH were linked in a buffered solution to furnish the sLe^a-KLH conjugate. The sLe^a concentration and sLe^a/KLH epitope ratio of the sLe^a conjugate were determined using resorcinol by measuring sialic acid content of sLe^a and KLH by a dye binding method (BioRad, Dye reagent) to be 874. The sLe^a-HSA conjugate (a target for ELISA) was prepared by reductive amination method similar to the method described for the preparation of STn-KLH conjugate [17].

Free sLe^a (unconjugated sLe^a), sLe^a-KLH conjugate and GPI-0100 were vialled separately. Mice were immunized with 5 µg of unconjugated sLe^a plus 100 µg GPI-0100, 5 µg of unconjugated sLe^a plus 20 µg KLH and 100 µg GPI-0100, KLH plus 100 µg GPI-0100, and 5 µg of sLe^a-KLH conjugate plus or minus GPI-0100, subcutaneously in 0.1 ml.

Animals and immunization

Six week old female C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Groups of five mice were immunized subcutaneously three times at 1-week intervals with different preparations mixed with 100 µg of GPI-0100 as indicated in Table 1. Mice were bled 7 days after the third immunization. A booster vaccination was administered 4 weeks after the third vaccination and mice were bled 7 days later. Mice were weighed at 24, 48, and 72 h after each vaccination.

ELISA

Enzyme-linked immunosorbent assays (ELISA) were performed as previously described [18]. sLe^a titers were measured by coating ELISA plates with 0.1 µg sLe^a-HSA per well. Serially diluted antisera or monoclonal antibody 19.9 were added to the wells and incubated for 1 h at room temperature. Goat anti-mouse IgM or IgG conjugated with alkaline phosphatase were used as secondary antibodies. The antibody titer was defined as the highest serum dilution showing an absorbance of 0.1 or greater over that of normal mouse (prevaccination) sera.

Flow cytometry

Cells from the ovarian cancer cell line SW626 and SCLC cell line DMS79 served as sLe^a positive targets and cells from the sLe^a negative melanoma cell line SK-MEL-28 served as negative control targets. Cells were incubated on ice with 20 µl of 1/20 diluted pre vaccination sera, post vaccination sera or murine mAb 121SLE (5 µg/ml) for 30 min [19]. After washing, 20 µl of 1/15 goat anti-mouse IgM or IgG labeled with fluorescein-isothiocyanate (FITC) was added, mixed and incubated for 30 min on ice. After washing, the positive population and mean fluorescence intensity (MFI) of the stained cells were analyzed by flow cytometry (FACScan, Becton and Dickinson, San Jose, CA). Pre and post vaccination sera were analyzed together and the pretreatment percent positive cells gaied at 10%. Results were considered positive when percent positive cells were threefold the pretreatment serum value and the MFI was 150% of the protectant serum MFI.

Complement dependent cytotoxicity (CDC)

Complement dependent cytotoxicity was assayed at a serum dilution of 1:4 with SW626 cells and human complement using a chromium release assay as described previously [20]. All assays were performed in triplicate. Controls included cells incubated only with culture median or complement. Spontaneous release was the chromium release by target cells incubated with complement alone and maximum release was the amount released by target cells after 2 h incubation in the presence of complement and 10% Triton X-100. The percent cytotoxicity was calculated according to the formula

$$\text{Specific release (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Dot blot immune stain

Dot blot immune staining of gangliosides and other structurally related antigens (listed in Table 2) were spotted on nitrocellulose strips. The unreacted sites were blocked with 3% HSA in PBS contain 0.05% Tween 20 for 2 h. Blocked strips were incubated with mouse sera diluted in 3% HSA-PBS at 1:150 dilution overnight at 4°C with gentle stir. The strips were washed with PBS containing 0.05% Tween 20 and incubated with anti-mouse Ig-horseradish peroxidase conjugate at 1:200 dilution for 2 h at room temperature. Then the strips were washed with PBS-0.05% Tween 20 and developed with developer solution contain 4-chloro-1-naphthol and hydrogen peroxide solution.

Results

Toxicity testing

There was no evidence of increased weight loss or other toxicity over the 7 weeks of followup in mice receiving the sLe^a-KLH conjugate plus 100 µg GPI-0100 compared to mice receiving GPI-0100 plus KLH or the other negative controls (data not shown). As in most groups of immunized mice, regardless of the antigen, there was 3–4% weight loss over the initial 2 days

after the initial vaccination, but this was followed by a rapid rebound and then normal weight gain there-after. One mouse in group 4 (mouse 4.5) was small to begin with, lost weight progressively and died before the forth vaccination. This mouse had malocclusion, a genetic defect. No evidence of toxicity has been seen in 15 other mice immunized more recently with this sLe^a-KLH plus saponin adjuvant vaccine. The failure to thrive of mouse 4.5 was not attributed to the vaccinations.

ELISA assays

The peak ELISA titers after four vaccinations are demonstrated in Table 1. No evidence of antibody induction was seen after immunization with sLe^a plus GPI-0100, KLH plus GPI-0100 or unconjugated sLe^a plus KLH plus GPI-0100. The sLe^a-KLH resulted in moderate elevations in ELISA reactivity which was significantly augmented when mixed with GPI-0100. Median antibody titers of 1/51,200 for IgG reactivity and 1/1,280 for IgM reactivity are higher than we have seen in previous studies with other carbohydrate antigens. Also unusual is that IgG antibody titers were significantly more elevated than the IgM antibody titers. This has been a consistent finding with the sLe^a-KLH conjugate vaccine in three separate experiments.

Cell surface reactivity

The results of flow cytometry testing on the SW626 ovarian cancer cell line for each of the five groups are summarized in Table 1 and results of the mice immunized with sLe^a-KLH plus GPI-0100 are demonstrated in Fig 3. The fifth mouse expired prior to the final (larger) bleed. Striking increases in IgM and IgG reactivity against SW626 were seen for all mice. Comparable cell surface reactivity was seen against a second sLe^a positive cell line (SCLC cell line DMS79). Median peak IgG MFIs for groups 1–5 against DMS79 were 4, 3, 3, 125 and 22, respectively (data not shown). No increase in reactivity against melanoma cell line SK-MEL-28 (which is sLe^a negative) was seen with the same sera. Cell surface reactivity against SW626 was also demonstrated by complement dependent cytotoxicity (CDC) assays using human complement and sera from the four mice in group 4 receiving four vaccinations with sLe^a-KLH plus GPI-0100 vaccines as demonstrated in Fig. 4. Once again no reactivity was detected prior to immunization and striking CDC reactivity was seen against SW626 after vaccination.

Specificity analysis

Specificity of the serologic reactivity induced by the sLe^a-KLH plus GPI-0100 vaccine was confirmed by standard dot blot assays. As demonstrated in Fig. 5, strong reactivity was detected against conjugates containing sLe^a but not those containing sLe^x, Le^x (from two different sources), Le^y, globo H, sTn, sTn clusters or trimers (c), Tn (c), GD3 ganglioside or polysialic acid. Specificity restricted to sLe^a was seen in all mice vaccinated with sLe^a-KLH plus GPI-0100. Positive control mAbs 121SLE against sLe^a and VK9 against globo H were appropriately positive Fig. 5.

Discussion

Previous studies have indicated the need for conjugation of purified carbohydrate or glycolipid antigens to a highly immunogenic carrier protein such as KLH plus the use of a potent adjuvant such as GPI-0100 for optimal antibody responses. We demonstrate this requirement here again. Vaccination with unconjugated sLe^a alone or with GPI-0100 or with GPI-0100 plus KLH resulted in no detectable antibody. Immunization with the sLe^a-KLH conjugate in the absence of GPI-0100 resulted in low or moderate titers of antibody which were significantly increased by the use of GPI-0100. The IgM and IgG antibodies induced against sLe^a were significantly high, higher than we have seen previously with comparable KLH conjugate vaccines against other carbohydrate and glycolipid antigens.

Despite the high levels of IgM and IgG antibodies induced against sLe^a and the known expression of sLe^a on a variety of normal murine tissues there was no obvious evidence of toxicity. There was minimal weight loss associated with the immunizations and the mice continued gaining weight over the following weeks suggesting that both the vaccine and the resulting high antibody titers against sLe^a were well tolerated. This lack of toxicity in the face of high titer antibody against a cell surface antigen expressed on a variety of tissues is consistent with results we have obtained previously. Antigens, though widely expressed, if expressed primarily or exclusively at the luminal borders of tissue, as is the case for sLe^a, have not been associated with toxicity after the administration of either antibody inducing vaccines or monoclonal antibodies (reviewed [15]).

There was a concern prior to initiating these experiments that antibodies induced by this sLe^a-KLH vaccine would cross react with a variety of other blood type-related antigens, including Le^x, sLe^x, sLe^y that are more widely expressed on normal tissues. It turned out that this was not the case. No antibodies induced by the sLe^a-KLH were detected against any of these antigens, other blood group related antigens including globo H, sTn and Tn, or GD3 or polysialic acid in dot blot assays. sLe^x and sLe^a have remarkably similar structures, differing from each other only in the location of the fucose and galactose linkages as indicated in Table 2. Le^x, Le^y, GD3 and globo H also have structural similarities to sLe^a but none were recognized by these high titer antibodies against sLe^a.

Antibodies have been shown to mediate therapeutic efficacy against tumors by antibody dependent cellular cytotoxicity (ADCC), by complement mediated cytotoxicity (CDC), or by other complement mediated mechanisms. ADCC requires IgG antibodies of the proper subclass, IgG 2 and 3 in mice and 1 and 3 in humans. These are the IgG subclasses that our KLH conjugate plus saponin adjuvant vaccines have induced in mice and patients in the past and that were induced in mice here with this sLe^a vaccine. Complement lysis of tumor cells requires antibodies that react with cell surface antigens which are in close proximity to the cell surface. We have demonstrated previously that monoclonal antibodies or vaccine induced antibodies in mice or patients that react with a number of cell surface carbohydrate antigens such as GM2, GD2, GD3, fucosyl GM1, Le^y or globo H, or cell surface globular proteins such as EPCAM (KSA), consistently induce complement mediated lysis. We have also demonstrated that antibodies of comparable titer, class and subclass reactive with cell surface mucin molecules such as MUC1, sTn, Tn and TF are unable to induce complement lysis, presumable as a consequence of the distance from the cell surface that complement is being activated [20]. sLe^a is expressed at the cell surface both as a glycolipid and as a glycoprotein primarily O-linked to mucin molecules. We demonstrate here significant complement lysis of the human ovarian cancer cell line SW626 with the sLe^a-KLH vaccine induced immune sera in the presence of human complement. This suggests that not all of the sLe^a on the surface of SW-626 is expressed on mucins. While protection or therapeutic tumor challenge experiments were not in the scope of the work described here, the target, sLe^a, and the antibodies induced are shown to be perfect for all of the antibody induced mechanisms known to have therapeutic impact.

We demonstrate here that this sLe^a-KLH plus saponin adjuvant vaccine induces high titers of antibodies that are specific for sLe^a, react with sLe^a expressing human cancer cells and mediate complement lysis in the presence of human complement. sLe^a is known to be abundantly expressed at the cell surface of cancers of GI origin and the majority of human SCLC and breast cancers. sLe^a expression on normal tissues, however, is restricted to the ductal epithelial tissues where it is expressed in a highly polar distribution primarily at the luminal borders, sites largely inaccessible to antibody mediated attack. These findings make sLe^a an appealing target for vaccination and suggest that this sLe^a-KLH conjugate vaccine is ready for testing in the clinic.

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Abbreviations

BSA	Bovine serum albumin
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescence-isothiocyanate
HSA	Human serum albumin
KLH	Keyhole limpet hemocyanin
MMCCH	4-(4- <i>N</i> -Maleimidomethyl) cyclohexane-1-carboxyl hydrazide
PBS	Phosphate buffered saline

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Fig. 1. Scheme for the synthesis of pentenyl glycoside of sLe^a hexasaccharide

Fig. 2.

Preparation of sLe^a-KLH conjugate involves ozone cleavage of sLe^a pentenyl glycoside and use of the heterobifunctional cross linker 4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide (MMCCH)

Fig. 3.

IgM and IgG FACS profiles for the five mice immunized with 5 µg sLe^a-KLH conjugate plus 100 µg GPI-0100 (group 4) tested on SW626 ovarian cancer cells. Median pre and post immunization results are shown. The percent positive cells (% of Max) and (MFI) and shown, "au" is arbitrary units of FITC positive cells

Fig. 4.

Complement dependant cytotoxicity results against SW626 using sera from mice immunized with 5 μ g sLe^a-KLH conjugate plus 100 μ g GPI-0100 (group 4) plus human complement. Sera was drawn pretreatment (*pre*) 1 week after the third vaccination (*post*), or 1 week after the forth vaccination (*boost*)

Fig. 5.
Dot blot immune stains for four of the five mice immunized with 5 µg sLe^a-KLH conjugate plus 100 µg GPI-0100 (group 4)

IgM or IgG antibody reciprocal titers after vaccination with sLe^a-KLH conjugate

Group	Mouse #	Antibody titer against SLe ^a	FACS on SW626 cells (% positive cell and MFI)			
			IgG	IgM	IgG (%)	MFI
5 µg SLe ^a alone Plus 100 µg GPI-0100	Pre	0	0	10	5	9
	1.1	0	0	9	4	17
	1.2	0	0	9	3	17
	1.3	0	0	7	4	17
	1.4	0	0	8	5	18
	1.5	0	0	8	3	17
	Median	0	0	8	4	17
	Pre	0	0	10	5	9
	2.1	0	0	11	3	24
	2.2	0	0	9	3	15
20 µg KLH alone Plus 100 µg GPI-0100	2.3	0	0	9	3	13
	2.4	0	0	8	3	10
	2.5	0	0	10	3	10
	Median	0	0	9	3	13
	Pre	0	0	10	5	9
	3.1	0	40	9	4	12
	3.2	0	0	9	3	15
	3.3	0	0	7	3	15
	3.4	0	0	6	2	13
	3.5	0	0	10	7	10
5 µg SLe ^a plus 20 µg KLH Plus 100 µg GPI-0100	Median	0	0	9	3	13
	Pre	0	0	10	5	9
	3.1	0	40	9	4	12
	3.2	0	0	9	3	15
	3.3	0	0	7	3	15
	3.4	0	0	6	2	13
	3.5	0	0	10	7	10
	Median	0	0	9	3	13
	Pre	0	0	10	5	9
	4.1	51,200	640	98	170	95
5 µg SLe ^a -KLH conjugate Plus 100 µg GPI-0100	4.2	51,200	5,120	98	233	99
	4.3	51,200	5,120	96	125	117
	4.4	51,200	1,280	68	13	53

Group	Mouse #	Antibody titer against SLe ^a		FACS on SW626 cells (% positive cell and MFI)			
		IgG	IgM	IgG (%)	MFI	IgM (%)	MFI
5 µg SLe ^a -KLH alone No adjuvant	4.5*	51,200	160	87	53	90	73
	Median	51,200	1,280	96	125	97	95
	Pre	0	0	10	5	10	9
	5.1	800	320	82	22	50	18
	5.2	800	160	83	22	56	27
	5.3	800	160	83	41	93	59
	5.4	200	160	77	22	42	19
	5.5	800	160	86	44	51	22
	Median	800	160	83	22	51	22

sLe^a-HSA was used as a target in ELISA and SW626 cells was used a target in FACS. Each value represents sera from an individual mouse drawn 7 days after the forth immunization

* Results for mouse 4.5 are from blood drawn 1 week after the third vaccination

Table 2Chemical structures of sLe^a and some related antigens

Sialyl Le ^a	NeuAcα2 → 3Galβ1 → 3(Fuca1→4)GlcNAcβ1 → 3Galβ1 -
Sialyl Le ^x	NeuAcα2 → 3Galβ1 → 3(Fuca1→3)GlcNAcβ1 → 3Galβ1 -
GD3	NeuAcα2 → 8NeuAcα2 → 3Galβ1 → 4G1cβ1 →1Cer
Le ^y	Fuca1→2 Galβ1 →4(Fuca1 →3) GlcNAcβ1 → 3Galβ1 -
Globo H	Fuca1→2Gal β 1→3GalNAc β 1→3Gal α 1→4 Galβ1 → 4Glcβ1 -
sTn	NeuAcα2 →6GalNAc α -O-Serine/Threonine
Tn	GalNAc α -O-Serine/Threonine

The structures with greatest similarity to sLe^a are highlighted in bold